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(54) **Expression cassettes for guard cell-preferential expression in plants**

(57) The present invention relates to expression cassettes comprising transcription regulating sequences with guard cell-preferential or guard cell-specific expression profiles in plants obtainable from *Arabidopsis thal-*

iana genomic DNA sequences as described by SEO ID NO: 1, 2 or 3.

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Description**FIELD OF THE INVENTION**

5 **[0001]** The present invention relates to expression cassettes comprising transcription regulating nucleotide sequences with guard cell-preferential or guard cell-specific expression profiles in plants obtainable from *Arabidopsis thaliana* gene At5g58580, or from Arabidopsis genomic DNA sequences as described by SEQ ID NO: 1, 2, 3, 4, 5, 6, 7, 8, 9, 12, 13, 14, or 15.

BACKGROUND OF THE INVENTION

10 **[0002]** Manipulation of plants to alter and/or improve phenotypic characteristics (such as productivity or quality) requires the expression of heterologous genes in plant tissues. Such genetic manipulation relies on the availability of a means to drive and to control gene expression as required. For example, genetic manipulation relies on the availability and use of suitable promoters which are effective in plants and which regulate gene expression so as to give the desired effect (s) in the transgenic plant.

15 **[0003]** The number of guard cell-specific or guard cell-preferential promoters described in the art is very limited (EP-A1 1 111 051; Plesch 2001). It is advantageous to have the choice of a variety of different promoters so that the most suitable promoter may be selected for a particular gene, construct, cell, tissue, plant or environment. Moreover, the increasing interest in cotransforming plants with multiple plant transcription units (PTU) and the potential problems associated with using common regulatory sequences for these purposes merit having a variety of promoter sequences available.

20 **[0004]** There is, therefore, a great need in the art for the identification of novel sequences that can be used for expression of selected transgenes in economically important plants. It is thus an objective of the present invention to provide new and alternative expression cassettes for guard cell-preferential or guard cell-specific expression of transgenes in plants. The objective is solved by the present invention.

SUMMARY OF THE INVENTION

25 **[0005]** Accordingly, a first embodiment of the invention relates to a expression cassette for guard cell-specific or guard cell-preferential transcription of an operatively linked nucleic acid sequences in plants comprising

i) at least one transcription regulating nucleotide sequence selected from the group consisting of

35 a) a transcription regulating nucleotide sequence of the plant gene described by the *Arabidopsis thaliana* genome locus At5g58580, and

b) the transcription regulating nucleotide sequences as described by SEQ ID NO: 1, 2, 3, 4, 5, 6, 7, 8, 9, 12, 13, 14 and 15, the functional equivalents thereof and the functional equivalent fragments thereof,

40 and functionally linked thereto

ii) at least one nucleic acid sequence which is heterologous in relation to said transcription regulating nucleotide sequence.

45 **[0006]** Preferably, the transcription regulating nucleotide sequence (or the functional equivalent thereof) is selected from the group of sequences consisting of

i) the sequences described by SEQ ID NOs: 1, 2, 3, 4, 5, 6, 7, 8, 9, 12, 13, 14 and 15,

50 ii) a fragment of at least 50 consecutive bases of a sequence under i) which has substantially the same promoter activity as the corresponding transcription regulating nucleotide sequence described by SEQ ID NO: 1, 2, 3, 4, 5, 6, 7, 8, 9, 12, 13, 14 or 15;

55 iii) a nucleotide sequence having substantial similarity (e.g., with a sequence identity of at least 40, 50, 60, to 70%, e.g., preferably 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, to 79%, generally at least 80%, e.g., 81% to 84%, at least 85%, e.g., 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, to 98% and 99%) to a transcription regulating nucleotide sequence described by SEQ ID NO: 1, 2, 3, 4, 5, 6, 7, 8, 9, 12, 13, 14 or 15;

iv) a nucleotide sequence capable of hybridizing (preferably under conditions equivalent to hybridization in 7%

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sodium dodecyl sulfate (SDS), 0.5 M NaPO₄, 1 mM EDTA at 50°C with washing in 2 X SSC, 0.1% SDS at 50°C (more desirably in 7% sodium dodecyl sulfate (SDS), 0.5 M NaPO₄, 1 mM EDTA at 50°C with washing in 1 X SSC, 0.1% SDS at 50°C, more desirably still in 7% sodium dodecyl sulfate (SDS), 0.5 M NaPO₄, 1 mM EDTA at 50°C with washing in 0.5 X SSC, 0.1% SDS at 50°C, preferably in 7% sodium dodecyl sulfate (SDS), 0.5 M NaPO₄, 1 mM EDTA at 50°C with washing in 0.1 X SSC, 0.1% SDS at 50°C, more preferably in 7% sodium dodecyl sulfate (SDS), 0.5 M NaPO₄, 1 mM EDTA at 50°C with washing in 0.1 X SSC, 0.1% SDS at 65°C) to a transcription regulating nucleotide sequence described by SEQ ID NO: 1, 2, 3, 4, 5, 6, 7, 8, 9, 12, 13, 14 or 15, or the complement thereof;

v) a nucleotide sequence capable of hybridizing (preferably under conditions equivalent to hybridization in 7% sodium dodecyl sulfate (SDS), 0.5 M NaPO₄, 1 mM EDTA at 50°C with washing in 2 X SSC, 0.1% SDS at 50°C (more desirably in 7% sodium dodecyl sulfate (SDS), 0.5 M NaPO₄, 1 mM EDTA at 50°C with washing in 1 X SSC, 0.1% SDS at 50°C, more desirably still in 7% sodium dodecyl sulfate (SDS), 0.5 M NaPO₄, 1 mM EDTA at 50°C with washing in 0.5 X SSC, 0.1% SDS at 50°C, preferably in 7% sodium dodecyl sulfate (SDS), 0.5 M NaPO₄, 1 mM EDTA at 50°C with washing in 0.1 X SSC, 0.1% SDS at 50°C, more preferably in 7% sodium dodecyl sulfate (SDS), 0.5 M NaPO₄, 1 mM EDTA at 50°C with washing in 0.1 X SSC, 0.1% SDS at 65°C) to a nucleic acid comprising 50 to 200 or more consecutive nucleotides of a transcription regulating nucleotide sequence described by SEQ ID NO: 1, 2, 3, 4, 5, 6, 7, 8, 9, 12, 13, 14 or 15, or the complement thereof;

vi) a nucleotide sequence which is the complement or reverse complement of any of the previously mentioned nucleotide sequences under i) to v).

[0007] A functional equivalent of the transcription regulating nucleotide sequence of the gene described by the locus At5g58580 is obtained or obtainable from plant genomic DNA from a gene encoding a polypeptide which has at least 70% amino acid sequence identity to a polypeptide selected from the group described by SEQ ID NO: 11, respectively.

[0008] The expression cassette may be employed for numerous expression purposes such as for example expression of a protein, or expression of an antisense RNA, sense or double-stranded RNA. Preferably, expression of the nucleic acid sequence confers to the plant an agronomically valuable trait.

[0009] Other embodiments of the invention relate to vectors comprising an expression cassette of the invention, and transgenic host cell or non-human organism comprising an expression cassette or a vector of the invention. Preferably the organism is a plant.

[0010] Another embodiment of the invention relates to a method for identifying and/or isolating a sequence with guard cell-specific or guard cell-preferential transcription regulating activity characterized that said identification and/or isolation utilizes a nucleic acid sequence encoding a amino acid sequence as described by SEQ ID NO: 11 or a part of at least 15 bases thereof. Preferably the nucleic acid sequences is described by SEQ ID NO: 10 or a part of at least 15 bases thereof. More preferably, identification and/or isolation is realized by a method selected from polymerase chain reaction, hybridization, and database screening.

[0011] Another embodiment of the invention relates to a method for providing a transgenic expression cassette for guard cell-specific or guard cell-preferential expression comprising the steps of:

I. isolating of a guard cell-preferential or guard cell-specific transcription regulating nucleotide sequence utilizing at least one nucleic acid sequence or a part thereof, wherein said sequence is encoding a polypeptide described by SEQ ID NO: 11, or a part of at least 15 bases thereof, and

II. functionally linking said guard cell-preferential or guard cell-specific transcription regulating nucleotide sequence to another nucleotide sequence of interest, which is heterologous in relation to said guard cell-preferential or guard cell-specific transcription regulating nucleotide sequence.

DEFINITIONS

[0012] It is to be understood that this invention is not limited to the particular methodology, protocols, cell lines, plant species or genera, constructs, and reagents described as such. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention which will be limited only by the appended claims. It must be noted that as used herein and in the appended claims, the singular forms "a," "and," and "the" include plural reference unless the context clearly dictates otherwise. Thus, for example, reference to "a vector" is a reference to one or more vectors and includes equivalents thereof known to those skilled in the art, and so forth.

[0013] The term "about" is used herein to mean approximately, roughly, around, or in the region of. When the term "about" is used in conjunction with a numerical range, it modifies that range by extending the boundaries above and

below the numerical values set forth. In general, the term "about" is used herein to modify a numerical value above and below the stated value by a variance of 20 per-cent up or down (higher or lower).

[0014] As used herein, the word "or" means any one member of a particular list and also includes any combination of members of that list.

[0015] The term "gene" is used broadly to refer to any segment of nucleic acid associated with a biological function. Thus, genes include coding sequences and/or the regulatory sequences required for their expression. For example, gene refers to a nucleic acid fragment that expresses mRNA or functional RNA, or encodes a specific protein, and which includes regulatory sequences. Genes also include non-expressed DNA segments that, for example, form recognition sequences for other proteins. Genes can be obtained from a variety of sources, including cloning from a source of interest or synthesizing from known or predicted sequence information, and may include sequences designed to have desired parameters.

[0016] The term "native" or "wild type" gene refers to a gene that is present in the genome of an untransformed cell, i.e., a cell not having a known mutation.

[0017] A "marker gene" encodes a selectable or screenable trait.

[0018] The term "chimeric gene" refers to any gene that contains

- 1) DNA sequences, including regulatory and coding sequences, that are not found together in nature, or
- 2) sequences encoding parts of proteins not naturally adjoined, or
- 3) parts of promoters that are not naturally adjoined.

[0019] Accordingly, a chimeric gene may comprise regulatory sequences and coding sequences that are derived from different sources, or comprise regulatory sequences and coding sequences derived from the same source, but arranged in a manner different from that found in nature.

[0020] A "transgene" refers to a gene that has been introduced into the genome by transformation and is stably maintained. Transgenes may include, for example, genes that are either heterologous or homologous to the genes of a particular plant to be transformed. Additionally, transgenes may comprise native genes inserted into a non-native organism, or chimeric genes. The term "endogenous gene" refers to a native gene in its natural location in the genome of an organism. A "foreign" gene refers to a gene not normally found in the host organism but that is introduced by gene transfer.

[0021] An "oligonucleotide" corresponding to a nucleotide sequence of the invention, e.g., for use in probing or amplification reactions, may be about 30 or fewer nucleotides in length (e.g., 9, 12, 15, 18, 20, 21 or 24, or any number between 9 and 30). Generally specific primers are upwards of 14 nucleotides in length. For optimum specificity and cost effectiveness, primers of 16 to 24 nucleotides in length may be preferred. Those skilled in the art are well versed in the design of primers for use processes such as PCR. If required, probing can be done with entire restriction fragments of the gene disclosed herein which may be 100's or even 1000's of nucleotides in length.

[0022] The terms "protein," "peptide" and "polypeptide" are used interchangeably herein.

[0023] The nucleotide sequences of the invention can be introduced into any plant. The genes to be introduced can be conveniently used in expression cassettes for introduction and expression in any plant of interest. Such expression cassettes will comprise the transcriptional initiation region of the invention linked to a nucleotide sequence of interest. Preferred promoters include constitutive, tissue-specific, developmental-specific, inducible and/or viral promoters, most preferred are the seed-specific or seed-preferential promoters of the invention. Such an expression cassette is provided with a plurality of restriction sites for insertion of the gene of interest to be under the transcriptional regulation of the regulatory regions. The expression cassette may additionally contain selectable marker genes. The cassette will include in the 5'-3' direction of transcription, a transcriptional and translational initiation region, a DNA sequence of interest, and a transcriptional and translational termination region functional in plants. The termination region may be native with the transcriptional initiation region, may be native with the DNA sequence of interest, or may be derived from another source. Convenient termination regions are available from the Ti-plasmid of *A. tumefaciens*, such, as the octopine synthase and nopaline synthase termination regions (see also, Guerineau 1991; Proudfoot 1991; Sanfacon 1991; Mogen 1990; Munroe 1990; Ballas 1989; Joshi 1987). "Coding sequence" refers to a DNA or RNA sequence that codes for a specific amino acid sequence and excludes the non-coding sequences. It may constitute an "uninterrupted coding sequence", i.e., lacking an intron, such as in a cDNA or it may include one or more introns bounded by appropriate splice junctions. An "intron" is a sequence of RNA which is contained in the primary transcript but which is removed through cleavage and re-ligation of the RNA within the cell to create the mature mRNA that can be translated into a protein.

[0024] The terms "open reading frame" and "ORF" refer to the amino acid sequence encoded between translation initiation and termination codons of a coding sequence. The terms "initiation codon" and "termination codon" refer to a unit of three adjacent nucleotides ('codon') in a coding sequence that specifies initiation and chain termination, respec-

tively, of protein synthesis (mRNA translation).

[0025] A "functional RNA" refers to an antisense RNA, ribozyme, or other RNA that is not translated.

[0026] The term "RNA transcript" refers to the product resulting from RNA polymerase catalyzed transcription of a DNA sequence. When the RNA transcript is a perfect complementary copy of the DNA sequence, it is referred to as the primary transcript or it may be a RNA sequence derived from posttranscriptional processing of the primary transcript and is referred to as the mature RNA. "Messenger RNA" (mRNA) refers to the RNA that is without introns and that can be translated into protein by the cell. "cDNA" refers to a single- or a double-stranded DNA that is complementary to and derived from mRNA.

[0027] "Transcription regulating nucleotide sequence", "regulatory sequences", and "suitable regulatory sequences", each refer to nucleotide sequences influencing the transcription, RNA processing or stability, or translation of the associated (or functionally linked) nucleotide sequence to be transcribed. The transcription regulating nucleotide sequence may have various localizations with the respect to the nucleotide sequences to be transcribed. The transcription regulating nucleotide sequence may be located upstream (5' non-coding sequences), within, or downstream (3' non-coding sequences) of the sequence to be transcribed (e.g., a coding sequence). The transcription regulating nucleotide sequences may be selected from the group comprising enhancers, promoters, translation leader sequences, introns, 5'-untranslated sequences, 3'-untranslated sequences, and polyadenylation signal sequences. They include natural and synthetic sequences as well as sequences, which may be a combination of synthetic and natural sequences. As is noted above, the term "transcription regulating nucleotide sequence" is not limited to promoters. However, preferably a transcription regulating nucleotide sequence of the invention comprises at least one promoter sequence (e.g., a sequence localized upstream of the transcription start of a gene capable to induce transcription of the downstream sequences). In one preferred embodiment the transcription regulating nucleotide sequence of the invention comprises the promoter sequence of the corresponding gene and - optionally and preferably - the native 5'-untranslated region of said gene. Furthermore, the 3'-untranslated region and/or the polyadenylation region of said gene may also be employed.

[0028] "5' non-coding sequence" refers to a nucleotide sequence located 5' (upstream) to the coding sequence. It is present in the fully processed mRNA upstream of the initiation codon and may affect processing of the primary transcript to mRNA, mRNA stability or translation efficiency (Turner 1995).

[0029] "3' non-coding sequence" refers to nucleotide sequences located 3' (downstream) to a coding sequence and include polyadenylation signal sequences and other sequences encoding regulatory signals capable of affecting mRNA processing or gene expression. The polyadenylation signal is usually characterized by affecting the addition of polyadenylic acid tracts to the 3' end of the mRNA precursor. The use of different 3' non-coding sequences is exemplified by Ingelbrecht et al., 1989.

[0030] The term "translation leader sequence" refers to that DNA sequence portion of a gene between the promoter and coding sequence that is transcribed into RNA and is present in the fully processed mRNA upstream (5') of the translation start codon. The translation leader sequence may affect processing of the primary transcript to mRNA, mRNA stability or translation efficiency.

[0031] "Signal peptide" refers to the amino terminal extension of a polypeptide, which is translated in conjunction with the polypeptide forming a precursor peptide and which is required for its entrance into the secretory pathway. The term "signal sequence" refers to a nucleotide sequence that encodes the signal peptide. The term "transit peptide" as used herein refers part of a expressed polypeptide (preferably to the amino terminal extension of a polypeptide), which is translated in conjunction with the polypeptide forming a precursor peptide and which is required for its entrance into a cell organelle (such as the plastids (e.g., chloroplasts) or mitochondria). The term "transit sequence" refers to a nucleotide sequence that encodes the transit peptide.

[0032] "Promoter" refers to a nucleotide sequence, usually upstream (5') to its coding sequence, which controls the expression of the coding sequence by providing the recognition for RNA polymerase and other factors required for proper transcription. "Promoter" includes a minimal promoter that is a short DNA sequence comprised of a TATA box and other sequences that serve to specify the site of transcription initiation, to which regulatory elements are added for control of expression. "Promoter" also refers to a nucleotide sequence that includes a minimal promoter plus regulatory elements that is capable of controlling the expression of a coding sequence or functional RNA. This type of promoter sequence consists of proximal and more distal upstream elements, the latter elements often referred to as enhancers. Accordingly, an "enhancer" is a DNA sequence which can stimulate promoter activity and may be an innate element of the promoter or a heterologous element inserted to enhance the level or tissue specificity of a promoter. It is capable of operating in both orientations (normal or flipped), and is capable of functioning even when moved either upstream or downstream from the promoter. Both enhancers and other upstream promoter elements bind sequence-specific DNA-binding proteins that mediate their effects. Promoters may be derived in their entirety from a native gene, or be composed of different elements, derived from different promoters found in nature, or even be comprised of synthetic DNA segments. A promoter may also contain DNA sequences that are involved in the binding of protein factors which control the effectiveness of transcription initiation in response to physiological or developmental conditions.

[0033] The "initiation site" is the position surrounding the first nucleotide that is part of the transcribed sequence, which

is also defined as position +1. With respect to this site all other sequences of the gene and its controlling regions are numbered. Downstream sequences (i.e., further protein encoding sequences in the 3' direction) are denominated positive, while upstream sequences (mostly of the controlling regions in the 5' direction) are denominated negative.

[0034] Promoter elements, particularly a TATA element, that are inactive or that have greatly reduced promoter activity in the absence of upstream activation are referred to as "minimal or core promoters." In the presence of a suitable transcription factor, the minimal promoter functions to permit transcription. A "minimal or core promoter" thus consists only of all basal elements needed for transcription initiation, e.g., a TATA box and/or an initiator.

[0035] "Constitutive expression" refers to expression using a constitutive or regulated promoter. "Conditional" and "regulated expression" refer to expression controlled by a regulated promoter.

[0036] "Constitutive promoter" refers to a promoter that is able to express the open reading frame (ORF) that it controls in all or nearly all of the plant tissues during all or nearly all developmental stages of the plant. Each of the transcription-activating elements do not exhibit an absolute tissue-specificity, but mediate transcriptional activation in most plant parts at a level of at least 1 % of the level reached in the part of the plant in which transcription is most active.

[0037] "Regulated promoter" refers to promoters that direct gene expression not constitutively, but in a temporally- and/or spatially-regulated manner, and includes both tissue-specific and inducible promoters. It includes natural and synthetic sequences as well as sequences which may be a combination of synthetic and natural sequences. Different promoters may direct the expression of a gene in different tissues or cell types, or at different stages of development, or in response to different environmental conditions. New promoters of various types useful in plant cells are constantly being discovered, numerous examples may be found in the compilation by Okamuro et al. (1989). Typical regulated promoters useful in plants include but are not limited to safener-inducible promoters, promoters derived from the tetracycline-inducible system, promoters derived from salicylate-inducible systems, promoters derived from alcohol-inducible systems, promoters derived from glucocorticoid-inducible system, promoters derived from pathogen-inducible systems, and promoters derived from ecdysone-inducible systems.

[0038] "Tissue-specific promoter" refers to regulated promoters that are not expressed in all plant cells but only in one or more cell types in specific organs (such as leaves or seeds), specific tissues (such as embryo or cotyledon), or specific cell types (such as leaf parenchyma, guard cells, or seed storage cells). These also include promoters that are temporally regulated, such as in early or late embryogenesis, during fruit ripening in developing seeds or fruit, in fully differentiated leaf, or at the onset of senescence.

[0039] "Inducible promoter" refers to those regulated promoters that can be turned on in one or more cell types by an external stimulus, such as a chemical, light, hormone, stress, or a pathogen.

[0040] "Operably-linked" or "functionally linked" refers preferably to the association of nucleic acid sequences on single nucleic acid fragment so that the function of one is affected by the other. For example, a regulatory DNA sequence is said to be "operably linked to" or "associated with" a DNA sequence that codes for an RNA or a polypeptide if the two sequences are situated such that the regulatory DNA sequence affects expression of the coding DNA sequence (i.e., that the coding sequence or functional RNA is under the transcriptional control of the promoter). Coding sequences can be operably-linked to regulatory sequences in sense or antisense orientation.

[0041] "Expression" refers to the transcription and/or translation of an endogenous gene, ORF or portion thereof, or a transgene in plants. For example, in the case of antisense constructs, expression may refer to the transcription of the antisense DNA only. In addition, expression refers to the transcription and stable accumulation of sense (mRNA) or functional RNA. Expression may also refer to the production of protein.

[0042] "Specific expression" is the expression of gene products which is limited to one or a few plant tissues (spatial limitation) and/or to one or a few plant developmental stages (temporal limitation). It is acknowledged that hardly a true specificity exists: promoters seem to be preferably switch on in some tissues, while in other tissues there can be no or only little activity. This phenomenon is known as leaky expression. However, with specific expression in this invention is meant preferable expression in one or a few plant tissues.

[0043] The "expression pattern" of a promoter (with or without enhancer) is the pattern of expression levels which shows where in the plant and in what developmental stage transcription is initiated by said promoter. Expression patterns of a set of promoters are said to be complementary when the expression pattern of one promoter shows little overlap with the expression pattern of the other promoter. The level of expression of a promoter can be determined by measuring the 'steady state' concentration of a standard transcribed reporter mRNA. This measurement is indirect since the concentration of the reporter mRNA is dependent not only on its synthesis rate, but also on the rate with which the mRNA is degraded. Therefore, the steady state level is the product of synthesis rates and degradation rates.

[0044] The rate of degradation can however be considered to proceed at a fixed rate when the transcribed sequences are identical, and thus this value can serve as a measure of synthesis rates. When promoters are compared in this way techniques available to those skilled in the art are hybridization S1-RNase analysis, northern blots and competitive RT-PCR. This list of techniques in no way represents all available techniques, but rather describes commonly used procedures used to analyze transcription activity and expression levels of mRNA.

[0045] The analysis of transcription start points in practically all promoters has revealed that there is usually no single

base at which transcription starts, but rather a more or less clustered set of initiation sites, each of which accounts for some start points of the mRNA. Since this distribution varies from promoter to promoter the sequences of the reporter mRNA in each of the populations would differ from each other. Since each mRNA species is more or less prone to degradation, no single degradation rate can be expected for different reporter mRNAs. It has been shown for various eukaryotic promoter sequences that the sequence surrounding the initiation site ('initiator') plays an important role in determining the level of RNA expression directed by that specific promoter. This includes also part of the transcribed sequences. The direct fusion of promoter to reporter sequences would therefore lead to suboptimal levels of transcription.

[0046] A commonly used procedure to analyze expression patterns and levels is through determination of the 'steady state' level of protein accumulation in a cell. Commonly used candidates for the reporter gene, known to those skilled in the art are beta-glucuronidase (GUS), chloramphenicol acetyl transferase (CAT) and proteins with fluorescent properties, such as green fluorescent protein (GFP) from *Aequora victoria*. In principle, however, many more proteins are suitable for this purpose, provided the protein does not interfere with essential plant functions. For quantification and determination of localization a number of tools are suited. Detection systems can readily be created or are available which are based on, e.g., immunochemical, enzymatic, fluorescent detection and quantification. Protein levels can be determined in plant tissue extracts or in intact tissue using *in situ* analysis of protein expression.

[0047] Generally, individual transformed lines with one chimeric promoter reporter construct will vary in their levels of expression of the reporter gene. Also frequently observed is the phenomenon that such transformants do not express any detectable product (RNA or protein). The variability in expression is commonly ascribed to 'position effects', although the molecular mechanisms underlying this inactivity are usually not clear.

[0048] "Overexpression" refers to the level of expression in transgenic cells or organisms that exceeds levels of expression in normal or untransformed (non-transgenic) cells or organisms.

[0049] "Antisense inhibition" refers to the production of antisense RNA transcripts capable of suppressing the expression of protein from an endogenous gene or a transgene.

[0050] "Gene silencing" refers to homology-dependent suppression of viral genes, transgenes, or endogenous nuclear genes. Gene silencing may be transcriptional, when the suppression is due to decreased transcription of the affected genes, or post-transcriptional, when the suppression is due to increased turnover (degradation) of RNA species homologous to the affected genes (English 1996). Gene silencing includes virus-induced gene silencing (Ruiz et al. 1998).

[0051] The terms "heterologous DNA sequence," "exogenous DNA segment" or "heterologous nucleic acid," as used herein, each refer to a sequence that originates from a source foreign to the particular host cell or, if from the same source, is modified from its original form. Thus, a heterologous gene in a host cell includes a gene that is endogenous to the particular host cell but has been modified through, for example, the use of DNA shuffling. The terms also include non-naturally occurring multiple copies of a naturally occurring DNA sequence. Thus, the terms refer to a DNA segment that is foreign or heterologous to the cell, or homologous to the cell but in a position within the host cell nucleic acid in which the element is not ordinarily found. Exogenous DNA segments are expressed to yield exogenous polypeptides.

A "homologous" DNA sequence is a DNA sequence that is naturally associated with a host cell into which it is introduced.

[0052] "Homologous to" in the context of nucleotide sequence identity refers to the similarity between the nucleotide sequence of two nucleic acid molecules or between the amino acid sequences of two protein molecules. Estimates of such homology are provided by either DNA-DNA or DNA-RNA hybridization under conditions of stringency as is well understood by those skilled in the art (as described in Haines and Higgins (eds.), *Nucleic Acid Hybridization*, IRL Press, Oxford, U.K.), or by the comparison of sequence similarity between two nucleic acids or proteins.

[0053] The term "substantially similar" refers to nucleotide and amino acid sequences that represent functional and/or structural equivalents of *Arabidopsis* sequences disclosed herein.

[0054] In its broadest sense, the term "substantially similar" when used herein with respect to a nucleotide sequence means that the nucleotide sequence is part of a gene which encodes a polypeptide having substantially the same structure and function as a polypeptide encoded by a gene for the reference nucleotide sequence, e.g., the nucleotide sequence comprises a promoter from a gene that is the ortholog of the gene corresponding to the reference nucleotide sequence, as well as promoter sequences that are structurally related the promoter sequences particularly exemplified herein, i.e., the substantially similar promoter sequences hybridize to the complement of the promoter sequences exemplified herein under high or very high stringency conditions. For example, altered nucleotide sequences which simply reflect the degeneracy of the genetic code but nonetheless encode amino acid sequences that are identical to a particular amino acid sequence are substantially similar to the particular sequences. The term "substantially similar" also includes nucleotide sequences wherein the sequence has been modified, for example, to optimize expression in particular cells, as well as nucleotide sequences encoding a variant polypeptide having one or more amino acid substitutions relative to the (unmodified) polypeptide encoded by the reference sequence, which substitution(s) does not alter the activity of the variant polypeptide relative to the unmodified polypeptide.

[0055] In its broadest sense, the term "substantially similar" when used herein with respect to polypeptide means that the polypeptide has substantially the same structure and function as the reference polypeptide. In addition, amino acid sequences that are substantially similar to a particular sequence are those wherein overall amino acid identity is at least

65% or greater to the instant sequences. Modifications that result in equivalent nucleotide or amino acid sequences are well within the routine skill in the art. The percentage of amino acid sequence identity between the substantially similar and the reference polypeptide is at least 65%, 66%, 67%, 68%, 69%, 70%, e.g., 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, and even 90% or more, e.g., 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, up to at least 99%, wherein the reference polypeptide is an *Arabidopsis* polypeptide encoded by a gene with a promoter having any one of SEQ ID NOs: 1, 2, 3, 4, 5, 6, 7, 8, 9, 12, 13, 14 or 15, a nucleotide sequence comprising an open reading frame having any one of SEQ ID NOs: 10, which encodes one of SEQ ID Nos: 11. One indication that two polypeptides are substantially similar to each other, besides having substantially the same function, is that an agent, e.g., an antibody, which specifically binds to one of the polypeptides, specifically binds to the other.

[0056] Sequence comparisons may be carried out using a Smith-Waterman sequence alignment algorithm (see e.g., Waterman 1995). The localS program, version 1.16, is preferably used with following parameters: match: 1, mismatch penalty: 0.33, open-gap penalty: 2, extended-gap penalty: 2.

[0057] Moreover, a nucleotide sequence that is "substantially similar" to a reference nucleotide sequence is said to be "equivalent" to the reference nucleotide sequence. The skilled artisan recognizes that equivalent nucleotide sequences encompassed by this invention can also be defined by their ability to hybridize, under low, moderate and/or stringent conditions (e.g., 0.1 X SSC, 0.1 % SDS, 65°C), with the nucleotide sequences that are within the literal scope of the instant claims.

[0058] What is meant by "substantially the same activity" when used in reference to a polynucleotide or polypeptide fragment is that the fragment has at least 65%, 66%, 67%, 68%, 69%, 70%, e.g., 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, and even 90% or more, e.g., 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, up to at least 99% of the activity of the full length polynucleotide or full length polypeptide.

[0059] "Target gene" refers to a gene on the replicon that expresses the desired target coding sequence, functional RNA, or protein. The target gene is not essential for replicon replication. Additionally, target genes may comprise native non-viral genes inserted into a non-native organism, or chimeric genes, and will be under the control of suitable regulatory sequences. Thus, the regulatory sequences in the target gene may come from any source, including the virus. Target genes may include coding sequences that are either heterologous or homologous to the genes of a particular plant to be transformed. However, target genes do not include native viral genes. Typical target genes include, but are not limited to genes encoding a structural protein, a seed storage protein, a protein that conveys herbicide resistance, and a protein that conveys insect resistance. Proteins encoded by target genes are known as "foreign proteins". The expression of a target gene in a plant will typically produce an altered plant trait.

[0060] The term "altered plant trait" means any phenotypic or genotypic change in a transgenic plant relative to the wild-type or non-transgenic plant host.

[0061] "Replication gene" refers to a gene encoding a viral replication protein. In addition to the ORF of the replication protein, the replication gene may also contain other overlapping or non-overlapping ORF(s), as are found in viral sequences in nature. While not essential for replication, these additional ORFs may enhance replication and/or viral DNA accumulation. Examples of such additional ORFs are AC3 and AL3 in ACMV and TGMV geminiviruses, respectively.

[0062] "Chimeric trans-acting replication gene" refers either to a replication gene in which the coding sequence of a replication protein is under the control of a regulated plant promoter other than that in the native viral replication gene, or a modified native viral replication gene, for example, in which a site specific sequence(s) is inserted in the 5' transcribed but untranslated region. Such chimeric genes also include insertion of the known sites of replication protein binding between the promoter and the transcription start site that attenuate transcription of viral replication protein gene.

[0063] "Chromosomally-integrated" refers to the integration of a foreign gene or DNA construct into the host DNA by covalent bonds. Where genes are not "chromosomally integrated" they may be "transiently expressed." Transient expression of a gene refers to the expression of a gene that is not integrated into the host chromosome but functions independently, either as part of an autonomously replicating plasmid or expression cassette, for example, or as part of another biological system such as a virus.

[0064] The term "transformation" refers to the transfer of a nucleic acid fragment into the genome of a host cell, resulting in genetically stable inheritance. Host cells containing the transformed nucleic acid fragments are referred to as "transgenic" cells, and organisms comprising transgenic cells are referred to as "transgenic organisms". Examples of methods of transformation of plants and plant cells include *Agrobacterium*-mediated transformation (De Blaere 1987) and particle bombardment technology (US 4,945,050).

[0065] Whole plants may be regenerated from transgenic cells by methods well known to the skilled artisan (see, for example, Fromm 1990).

[0066] "Transformed," "transgenic," and "recombinant" refer to a host organism such as a bacterium or a plant into which a heterologous nucleic acid molecule has been introduced. The nucleic acid molecule can be stably integrated into the genome generally known in the art and are disclosed (Sambrook 1989; Innis 1995; Gelfand 1995; Innis & Gelfand 1999. Known methods of PCR include, but are not limited to, methods using paired primers, nested primers, single specific primers, degenerate primers, gene-specific primers, vector-specific primers, partially mismatched primers, and

the like. For example, "transformed," "transformant," and "transgenic" plants or calli have been through the transformation process and contain a foreign gene integrated into their chromosome. The term "untransformed" refers to normal plants that have not been through the transformation process.

[0067] "Transiently transformed" refers to cells in which transgenes and foreign DNA have been introduced (for example, by such methods as *Agrobacterium*-mediated transformation or biolistic bombardment), but not selected for stable maintenance.

[0068] "Stably transformed" refers to cells that have been selected and regenerated on a selection media following transformation.

[0069] "Transient expression" refers to expression in cells in which a virus or a transgene is introduced by viral infection or by such methods as *Agrobacterium*-mediated transformation, electroporation, or biolistic bombardment, but not selected for its stable maintenance.

[0070] "Genetically stable" and "heritable" refer to chromosomally-integrated genetic elements that are stably maintained in the plant and stably inherited by progeny through successive generations.

[0071] "Primary transformant" and "T0 generation" refer to transgenic plants that are of the same genetic generation as the tissue which was initially transformed (i.e., not having gone through meiosis and fertilization since transformation).

[0072] "Secondary transformants" and the "T1, T2, T3, etc. generations" refer to transgenic plants derived from primary transformants through one or more meiotic and fertilization cycles. They may be derived by self-fertilization of primary or secondary transformants or crosses of primary or secondary transformants with other transformed or untransformed plants.

[0073] "Wild-type" refers to a virus or organism found in nature without any known mutation.

[0074] "Genome" refers to the complete genetic material of an organism.

[0075] The term "nucleic acid" refers to deoxyribonucleotides or ribonucleotides and polymers thereof in either single- or double-stranded form, composed of monomers (nucleotides) containing a sugar, phosphate and a base which is either a purine or pyrimidine. Unless specifically limited, the term encompasses nucleic acids containing known analogs of natural nucleotides which have similar binding properties as the reference nucleic acid and are metabolized in a manner similar to naturally occurring nucleotides. Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (e.g., degenerate codon substitutions) and complementary sequences as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzer 1991; Ohtsuka 1985; Rossolini 1994). A "nucleic acid fragment" is a fraction of a given nucleic acid molecule. In higher plants, deoxyribonucleic acid (DNA) is the genetic material while ribonucleic acid (RNA) is involved in the transfer of information contained within DNA into proteins. The term "nucleotide sequence" refers to a polymer of DNA or RNA which can be single- or double-stranded, optionally containing synthetic, non-natural or altered nucleotide bases capable of incorporation into DNA or RNA polymers. The terms "nucleic acid" or "nucleic acid sequence" may also be used interchangeably with gene, cDNA, DNA and RNA encoded by a gene.

[0076] The invention encompasses isolated or substantially purified nucleic acid or protein compositions. In the context of the present invention, an "isolated" or "purified" DNA molecule or an "isolated" or "purified" polypeptide is a DNA molecule or polypeptide that, by the hand of man, exists apart from its native environment and is therefore not a product of nature. An isolated DNA molecule or polypeptide may exist in a purified form or may exist in a non-native environment such as, for example, a transgenic host cell. For example, an "isolated" or "purified" nucleic acid molecule or protein, or biologically active portion thereof, is substantially free of other cellular material, or culture medium when produced by recombinant techniques, or substantially free of chemical precursors or other chemicals when chemically synthesized. Preferably, an "isolated" nucleic acid is free of sequences (preferably protein encoding sequences) that naturally flank the nucleic acid (i.e., sequences located at the 5' and 3' ends of the nucleic acid) in the genomic DNA of the organism from which the nucleic acid is derived. For example, in various embodiments, the isolated nucleic acid molecule can contain less than about 5 kb, 4 kb, 3 kb, 2 kb, 1 kb, 0.5 kb, or 0.1 kb of nucleotide sequences that naturally flank the nucleic acid molecule in genomic DNA of the cell from which the nucleic acid is derived. A protein that is substantially free of cellular material includes preparations of protein or polypeptide having less than about 30%, 20%, 10%, 5%, (by dry weight) of contaminating protein. When the protein of the invention, or biologically active portion thereof, is recombinantly produced, preferably culture medium represents less than about 30%, 20%, 10%, or 5% (by dry weight) of chemical precursors or non-protein of interest chemicals.

[0077] The nucleotide sequences of the invention include both the naturally occurring sequences as well as mutant (variant) forms. Such variants will continue to possess the desired activity, i.e., either promoter activity or the activity of the product encoded by the open reading frame of the non-variant nucleotide sequence.

[0078] The term "variant" with respect to a sequence (e.g., a polypeptide or nucleic acid sequence such as - for example - a transcription regulating nucleotide sequence of the invention) is intended to mean substantially similar sequences. For nucleotide sequences comprising an open reading frame, variants include those sequences that, because of the degeneracy of the genetic code, encode the identical amino acid sequence of the native protein. Naturally occurring

allelic variants such as these can be identified with the use of well-known molecular biology techniques, as, for example, with polymerase chain reaction (PCR) and hybridization techniques. Variant nucleotide sequences also include synthetically derived nucleotide sequences, such as those generated, for example, by using site-directed mutagenesis and for open reading frames, encode the native protein, as well as those that encode a polypeptide having amino acid substitutions relative to the native protein. Generally, nucleotide sequence variants of the invention will have at least 40, 50, 60, to 70%, e.g., preferably 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, to 79%, generally at least 80%, e.g., 81%-84%, at least 85%, e.g., 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, to 98% and 99% nucleotide sequence identity to the native (wild type or endogenous) nucleotide sequence.

[0079] "Conservatively modified variations" of a particular nucleic acid sequence refers to those nucleic acid sequences that encode identical or essentially identical amino acid sequences, or where the nucleic acid sequence does not encode an amino acid sequence, to essentially identical sequences. Because of the degeneracy of the genetic code, a large number of functionally identical nucleic acids encode any given polypeptide. For instance the codons CGT, CGC, CGA, CGG, AGA, and AGG all encode the amino acid arginine. Thus, at every position where an arginine is specified by a codon, the codon can be altered to any of the corresponding codons described without altering the encoded protein. Such nucleic acid variations are "silent variations" which are one species of "conservatively modified variations." Every nucleic acid sequence described herein which encodes a polypeptide also describes every possible silent variation, except where otherwise noted. One of skill will recognize that each codon in a nucleic acid (except ATG, which is ordinarily the only codon for methionine) can be modified to yield a functionally identical molecule by standard techniques. Accordingly, each "silent variation" of a nucleic acid which encodes a polypeptide is implicit in each described sequence.

[0080] The nucleic acid molecules of the invention can be "optimized" for enhanced expression in plants of interest (see, for example, WO 91/16432; Perlak 1991; Murray 1989). In this manner, the open reading frames in genes or gene fragments can be synthesized utilizing plant-preferred codons (see, for example, Campbell & Gowri, 1990 for a discussion of host-preferred codon usage). Thus, the nucleotide sequences can be optimized for expression in any plant. It is recognized that all or any part of the gene sequence may be optimized or synthetic. That is, synthetic or partially optimized sequences may also be used. Variant nucleotide sequences and proteins also encompass, sequences and protein derived from a mutagenic and recombinogenic procedure such as DNA shuffling. With such a procedure, one or more different coding sequences can be manipulated to create a new polypeptide possessing the desired properties. In this manner, libraries of recombinant polynucleotides are generated from a population of related sequence polynucleotides comprising sequence regions that have substantial sequence identity and can be homologously recombined in vitro or in vivo. Strategies for such DNA shuffling are known in the art (see, for example, Stemmer 1994; Stemmer 1994; Cramer 1997; Moore 1997; Zhang 1997; Cramer 1998; and US 5,605,793 and 5,837,458).

[0081] By "variant" polypeptide is intended a polypeptide derived from the native protein by deletion (so-called truncation) or addition of one or more amino acids to the N-terminal and/or C-terminal end of the native protein; deletion or addition of one or more amino acids at one or more sites in the native protein; or substitution of one or more amino acids at one or more sites in the native protein. Such variants may result from, for example, genetic polymorphism or from human manipulation. Methods for such manipulations are generally known in the art.

[0082] Thus, the polypeptides may be altered in various ways including amino acid substitutions, deletions, truncations, and insertions. Methods for such manipulations are generally known in the art. For example, amino acid sequence variants of the polypeptides can be prepared by mutations in the DNA. Methods for mutagenesis and nucleotide sequence alterations are well known in the art (see, for example, Kunkel 1985; Kunkel 1987; US 4,873,192; Walker & Gaastra, 1983 and the references cited therein). Guidance as to appropriate amino acid substitutions that do not affect biological activity of the protein of interest may be found in the model of Dayhoff et al. (1978). Conservative substitutions, such as exchanging one amino acid with another having similar properties, are preferred.

[0083] Individual substitutions deletions or additions that alter, add or delete a single amino acid or a small percentage of amino acids (typically less than 5%, more typically less than 1 %) in an encoded sequence are "conservatively modified variations," where the alterations result in the substitution of an amino acid with a chemically similar amino acid. Conservative substitution tables providing functionally similar amino acids are well known in the art. The following five groups each contain amino acids that are conservative substitutions for one another: Aliphatic: Glycine (G), Alanine (A), Valine (V), Leucine (L), Isoleucine (I); Aromatic: Phenylalanine (F), Tyrosine (Y), Tryptophan (W); Sulfur-containing: Methionine (M), Cysteine (C); Basic: Arginine (R), Lysine (K), Histidine (H); Acidic: Aspartic acid (D), Glutamic acid (E), Asparagine (N), Glutamine (Q). See also, Creighton, 1984. In addition, individual substitutions, deletions or additions which alter, add or delete a single amino acid or a small percentage of amino acids in an encoded sequence are also "conservatively modified variations."

[0084] "Expression cassette" as used herein means a DNA sequence capable of directing expression of a particular nucleotide sequence in an appropriate host cell, comprising a promoter operably linked to a nucleotide sequence of interest, which is - optionally - operably linked to termination signals and/or other regulatory elements. An expression cassette may also comprise sequences required for proper translation of the nucleotide sequence. The coding region usually codes for a protein of interest but may also code for a functional RNA of interest, for example antisense RNA or

a nontranslated RNA, in the sense or antisense direction. The expression cassette comprising the nucleotide sequence of interest may be chimeric, meaning that at least one of its components is heterologous with respect to at least one of its other components. The expression cassette may also be one, which is naturally occurring but has been obtained in a recombinant form useful for heterologous expression. An expression cassette may be assembled entirely extracellularly (e.g., by recombinant cloning techniques). However, an expression cassette may also be assembled using in part endogenous components. For example, an expression cassette may be obtained by placing (or inserting) a promoter sequence upstream of an endogenous sequence, which thereby becomes functionally linked and controlled by said promoter sequences. Likewise, a nucleic acid sequence to be expressed may be placed (or inserted) downstream of an endogenous promoter sequence thereby forming an expression cassette. The expression of the nucleotide sequence in the expression cassette may be under the control of a constitutive promoter or of an inducible promoter which initiates transcription only when the host cell is exposed to some particular external stimulus. In the case of a multicellular organism, the promoter can also be specific to a particular tissue or organ or stage of development (e.g., the guard-cell specific or guard-cell preferential promoters of the invention).

[0085] "Vector" is defined to include, inter alia, any plasmid, cosmid, phage or Agrobacterium binary vector in double or single stranded linear or circular form which may or may not be self transmissible or mobilizable, and which can transform prokaryotic or eukaryotic host either by integration into the cellular genome or exist extrachromosomally (e.g. autonomous replicating plasmid with an origin of replication).

[0086] Specifically included are shuttle vectors by which is meant a DNA vehicle capable, naturally or by design, of replication in two different host organisms, which may be selected from actinomycetes and related species, bacteria and eukaryotic (e.g. higher plant, mammalian, yeast or fungal cells).

[0087] Preferably the nucleic acid in the vector is under the control of, and operably linked to, an appropriate promoter or other regulatory elements for transcription in a host cell such as a microbial, e.g. bacterial, or plant cell. The vector may be a bi-functional expression vector which functions in multiple hosts. In the case of genomic DNA, this may contain its own promoter or other regulatory elements and in the case of cDNA this may be under the control of an appropriate promoter or other regulatory elements for expression in the host cell.

[0088] "Cloning vectors" typically contain one or a small number of restriction endonuclease recognition sites at which foreign DNA sequences can be inserted in a determinable fashion without loss of essential biological function of the vector, as well as a marker gene that is suitable for use in the identification and selection of cells transformed with the cloning vector. Marker genes typically include genes that provide tetracycline resistance, hygromycin resistance or ampicillin resistance.

[0089] A "transgenic plant" is a plant having one or more plant cells that contain an expression vector.

[0090] "Plant tissue" includes differentiated and undifferentiated tissues or plants, including but not limited to roots, stems, shoots, leaves, pollen, seeds, tumor tissue and various forms of cells and culture such as single cells, protoplast, embryos, and callus tissue. The plant tissue may be in plants or in organ, tissue or cell culture.

[0091] The following terms are used to describe the sequence relationships between two or more nucleic acids or polynucleotides: (a) "reference sequence", (b) "comparison window", (c) "sequence identity", (d) "percentage of sequence identity", and (e) "substantial identity".

(a) As used herein, "reference sequence" is a defined sequence used as a basis for sequence comparison. A reference sequence may be a subset or the entirety of a specified sequence; for example, as a segment of a full length cDNA or gene sequence, or the complete cDNA or gene sequence.

(b) As used herein, "comparison window" makes reference to a contiguous and specified segment of a polynucleotide sequence, wherein the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. Generally, the comparison window is at least 20 contiguous nucleotides in length, and optionally can be 30, 40, 50, 100, or longer. Those of skill in the art understand that to avoid a high similarity to a reference sequence due to inclusion of gaps in the polynucleotide sequence a gap penalty is typically introduced and is subtracted from the number of matches.

Methods of alignment of sequences for comparison are well known in the art. Thus, the determination of percent identity between any two sequences can be accomplished using a mathematical algorithm. Preferred, non-limiting examples of such mathematical algorithms are the algorithm of Myers and Miller, 1988; the local homology algorithm of Smith et al. 1981; the homology alignment algorithm of Needleman and Wunsch 1970; the search-for-similarity-method of Pearson and Lipman 1988; the algorithm of Karlin and Altschul, 1990, modified as in Karlin and Altschul, 1993.

Computer implementations of these mathematical algorithms can be utilized for comparison of sequences to determine sequence identity. Such implementations include, but are not limited to: CLUSTAL in the PC/Gene program (available from Intelligenetics, Mountain View, Calif.); the ALIGN program (Version 2.0) and GAP, BESTFIT, BLAST,

FASTA, and TFASTA in the Wisconsin Genetics Software Package, Version 8 (available from Genetics Computer Group (GCG), 575 Science Drive, Madison, Wis., USA). Alignments using these programs can be performed using the default parameters. The CLUSTAL program is well described (Higgins 1988, 1989; Corpet 1988; Huang 1992; Pearson 1994). The ALIGN program is based on the algorithm of Myers and Miller, supra. The BLAST programs of Altschul et al., 1990, are based on the algorithm of Karlin and Altschul supra.

Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>). This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul 1990). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are then extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always >0) and N (penalty score for mismatching residues; always <0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when the cumulative alignment score falls off by the quantity X from its maximum achieved value, the cumulative score goes to zero or below due to the accumulation of one or more negative-scoring residue alignments, or the end of either sequence is reached.

In addition to calculating percent sequence identity, the BLAST algorithm also performs a statistical analysis of the similarity between two sequences (see, e.g., Karlin & Altschul (1993)). One measure of similarity provided by the BLAST algorithm is the smallest sum probability ($P(N)$), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a test nucleic acid sequence is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid sequence to the reference nucleic acid sequence is less than about 0.1, more preferably less than about 0.01, and most preferably less than about 0.001.

To obtain gapped alignments for comparison purposes, Gapped BLAST (in BLAST 2.0) can be utilized as described in Altschul et al. 1997. Alternatively, PSI-BLAST (in BLAST 2.0) can be used to perform an iterated search that detects distant relationships between molecules. See Altschul et al., supra. When utilizing BLAST, Gapped BLAST, PSI-BLAST, the default parameters of the respective programs (e.g. BLASTN for nucleotide sequences, BLASTX for proteins) can be used. The BLASTN program (for nucleotide sequences) uses as defaults a word length (W) of 11, an expectation (E) of 10, a cutoff of 100, $M=5$, $N=-4$, and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a word length (W) of 3, an expectation (E) of 10, and the BLOSUM62 scoring matrix (see Henikoff & Henikoff, 1989). See <http://www.ncbi.nlm.nih.gov>. Alignment may also be performed manually by inspection.

For purposes of the present invention, comparison of nucleotide sequences for determination of percent sequence identity to the promoter sequences disclosed herein is preferably made using the BlastN program (version 1.4.7 or later) with its default parameters or any equivalent program. By "equivalent program" is intended any sequence comparison program that, for any two sequences in question, generates an alignment having identical nucleotide or amino acid residue matches and an identical percent sequence identity when compared to the corresponding alignment generated by the preferred program.

(c) As used herein, "sequence identity" or "identity" in the context of two nucleic acid or polypeptide sequences makes reference to the residues in the two sequences that are the same when aligned for maximum correspondence over a specified comparison window. When percentage of sequence identity is used in reference to proteins it is recognized that residue positions which are not identical often differ by conservative amino acid substitutions, where amino acid residues are substituted for other amino acid residues with similar chemical properties (e.g., charge or hydrophobicity) and therefore do not change the functional properties of the molecule. When sequences differ in conservative substitutions, the percent sequence identity may be adjusted upwards to correct for the conservative nature of the substitution. Sequences that differ by such conservative substitutions are said to have "sequence similarity" or "similarity." Means for making this adjustment are well known to those of skill in the art. Typically this involves scoring a conservative substitution as a partial rather than a full mismatch, thereby increasing the percentage sequence identity. Thus, for example, where an identical amino acid is given a score of 1 and a non-conservative substitution is given a score of zero, a conservative substitution is given a score between zero and 1. The scoring of conservative substitutions is calculated, e.g., as implemented in the program PC/GENE (Intelligenetics, Mountain View, Calif.).

(d) As used herein, "percentage of sequence identity" means the value determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which does not

comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison, and multiplying the result by 100 to yield the percentage of sequence identity.

(e) (i) The term "substantial identity" or "substantial similarity" of polynucleotide sequences (preferably for a protein encoding sequence) means that a polynucleotide comprises a sequence that has at least 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, or 79%, preferably at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, or 89%, more preferably at least 90%, 91%, 92%, 93%, or 94%, and most preferably at least 95%, 96%, 97%, 98%, or 99% sequence identity, compared to a reference sequence using one of the alignment programs described using standard parameters. The term "substantial identity" or "substantial similarity" of polynucleotide sequences (preferably for promoter sequence) means (as described above for variants) that a polynucleotide comprises a sequence that has at least 40, 50, 60, to 70%, e.g., preferably 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, to 79%, generally at least 80%, e.g., 81%-84%, at least 85%, e.g., 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, to 98% and 99% nucleotide sequence identity compared to a reference sequence using one of the alignment programs described using standard parameters. One of skill in the art will recognize that these values can be appropriately adjusted to determine corresponding identity of proteins encoded by two nucleotide sequences by taking into account codon degeneracy, amino acid similarity, reading frame positioning, and the like. Substantial identity of amino acid sequences for these purposes normally means sequence identity of at least 70%, more preferably at least 80%, 90%, and most preferably at least 95%.

Another indication that nucleotide sequences are substantially identical is if two molecules hybridize to each other under stringent conditions (see below). Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. However, stringent conditions encompass temperatures in the range of about 1 °C to about 20°C, depending upon the desired degree of stringency as otherwise qualified herein. Nucleic acids that do not hybridize to each other under stringent conditions are still substantially identical if the polypeptides they encode are substantially identical. This may occur, e.g., when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code. One indication that two nucleic acid sequences are substantially identical is when the polypeptide encoded by the first nucleic acid is immunologically cross reactive with the polypeptide encoded by the second nucleic acid.

(ii) The term "substantial identity" in the context of a peptide indicates that a peptide comprises a sequence with at least 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, or 79%, preferably 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, or 89%, more preferably at least 90%, 91%, 92%, 93%, or 94%, or even more preferably, 95%, 96%, 97%, 98% or 99%, sequence identity to the reference sequence over a specified comparison window. Preferably, optimal alignment is conducted using the homology alignment algorithm of Needleman and Wunsch (1970). An indication that two peptide sequences are substantially identical is that one peptide is immunologically reactive with antibodies raised against the second peptide. Thus, a peptide is substantially identical to a second peptide, for example, where the two peptides differ only by a conservative substitution.

[0092] For sequence comparison, typically one sequence acts as a reference sequence to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are input into a computer, subsequence coordinates are designated if necessary, and sequence algorithm program parameters are designated. The sequence comparison algorithm then calculates the percent sequence identity for the test sequence(s) relative to the reference sequence, based on the designated program parameters.

[0093] As noted above, another indication that two nucleic acid sequences are substantially identical is that the two molecules hybridize to each other under stringent conditions. The phrase "hybridizing specifically to" refers to the binding, duplexing, or hybridizing of a molecule only to a particular nucleotide sequence under stringent conditions when that sequence is present in a complex mixture (e.g., total cellular) DNA or RNA. "Bind(s) substantially" refers to complementary hybridization between a probe nucleic acid and a target nucleic acid and embraces minor mismatches that can be accommodated by reducing the stringency of the hybridization media to achieve the desired detection of the target nucleic acid sequence.

[0094] "Stringent hybridization conditions" and "stringent hybridization wash conditions" in the context of nucleic acid hybridization experiments such as Southern and Northern hybridization are sequence dependent, and are different under different environmental parameters. The T_m is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe. Specificity is typically the function of post-hybridization washes, the critical factors being the ionic strength and temperature of the final wash solution. For DNA-DNA hybrids, the T_m can be approximated from the equation of Meinkoth and Wahl, 1984:

$$T_m = 81.5^{\circ}\text{C} + 16.6 (\log_{10} M) + 0.41 (\%GC) - 0.61 (\% \text{ form}) - 500 / L$$

5 where M is the molarity of monovalent cations, %GC is the percentage of guanosine and cytosine nucleotides in the DNA, % form is the percentage of formamide in the hybridization solution, and L is the length of the hybrid in base pairs. T_m is reduced by about 1°C for each 1% of mismatching; thus, T_m , hybridization, and/or wash conditions can be adjusted to hybridize to sequences of the desired identity. For example, if sequences with >90% identity are sought, the T_m can be decreased 10°C. Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point I for the specific sequence and its complement at a defined ionic strength and pH. However, severely stringent conditions can utilize a hybridization and/or wash at 1, 2, 3, or 4°C lower than the thermal melting point I; moderately stringent conditions can utilize a hybridization and/or wash at 6, 7, 8, 9, or 10°C lower than the thermal melting point I; low stringency conditions can utilize a hybridization and/or wash at 11, 12, 13, 14, 15, or 20°C lower than the thermal melting point I. Using the equation, hybridization and wash compositions, and desired T, those of ordinary skill will understand that variations in the stringency of hybridization and/or wash solutions are inherently described. If the desired degree of mismatching results in a T of less than 45°C (aqueous solution) or 32°C (formamide solution), it is preferred to increase the SSC concentration so that a higher temperature can be used. An extensive guide to the hybridization of nucleic acids is found in Tijssen, 1993. Generally, highly stringent hybridization and wash conditions are selected to be about 5°C lower than the thermal melting point T_m for the specific sequence at a defined ionic strength and pH.

20 **[0095]** An example of highly stringent wash conditions is 0.15 M NaCl at 72°C for about 15 minutes. An example of stringent wash conditions is a 0.2 X SSC wash at 65°C for 15 minutes (see, Sambrook, *infra*, for a description of SSC buffer). Often, a high stringency wash is preceded by a low stringency wash to remove background probe signal. An example medium stringency wash for a duplex of, e.g., more than 100 nucleotides, is 1 X SSC at 45°C for 15 minutes. An example low stringency wash for a duplex of, e.g., more than 100 nucleotides, is 4 to 6 X SSC at 40°C for 15 minutes. For short probes (e.g., about 10 to 50 nucleotides), stringent conditions typically involve salt concentrations of less than about 1.5 M, more preferably about 0.01 to 1.0 M, Na ion concentration (or other salts) at pH 7.0 to 8.3, and the temperature is typically at least about 30°C and at least about 60°C for long robes (e.g., >50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. In general, a signal to noise ratio of 2 X (or higher) than that observed for an unrelated probe in the particular hybridization assay indicates detection of a specific hybridization. Nucleic acids that do not hybridize to each other under stringent conditions are still substantially identical if the proteins that they encode are substantially identical. This occurs, e.g., when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code.

35 **[0096]** Very stringent conditions are selected to be equal to the T_m for a particular probe. An example of stringent conditions for hybridization of complementary nucleic acids which have more than 100 complementary residues on a filter in a Southern or Northern blot is 50% formamide, e.g., hybridization in 50% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 0.1 x SSC at 60 to 65°C. Exemplary low stringency conditions include hybridization with a buffer solution of 30 to 35% formamide, 1 M NaCl, 1% SDS (sodium dodecyl sulphate) at 37°C, and a wash in 1 X to 2 X SSC (20 X SSC=3.0 M NaCl/0.3 M trisodium citrate) at 50 to 55°C. Exemplary moderate stringency conditions include hybridization in 40 to 45% formamide, 1.0 M NaCl, 1% SDS at 37°C, and a wash in 0.5 X to 1 X SSC at 55 to 60°C.

40 **[0097]** The following are examples of sets of hybridization/wash conditions that may be used to clone orthologous nucleotide sequences that are substantially identical to reference nucleotide sequences of the present invention: a reference nucleotide sequence preferably hybridizes to the reference nucleotide sequence in 7% sodium dodecyl sulfate (SDS), 0.5 M NaPO₄, 1 mM EDTA at 50°C with washing in 2 X SSC, 0.1% SDS at 50°C, more desirably in 7% sodium dodecyl sulfate (SDS), 0.5 M NaPO₄, 1 mM EDTA at 50°C with washing in 1 X SSC, 0.1% SDS at 50°C, more desirably still in 7% sodium dodecyl sulfate (SDS), 0.5 M NaPO₄, 1 mM EDTA at 50°C with washing in 0.5 X SSC, 0.1% SDS at 50°C, preferably in 7% sodium dodecyl sulfate (SDS), 0.5 M NaPO₄, 1 mM EDTA at 50°C with washing in 0.1 X SSC, 0.1% SDS at 50°C, more preferably in 7% sodium dodecyl sulfate (SDS), 0.5 M NaPO₄, 1 mM EDTA at 50°C with washing in 0.1 X SSC, 0.1% SDS at 65°C.

50 **[0098]** "DNA shuffling" is a method to introduce mutations or rearrangements, preferably randomly, in a DNA molecule or to generate exchanges of DNA sequences between two or more DNA molecules, preferably randomly. The DNA molecule resulting from DNA shuffling is a shuffled DNA molecule that is a non-naturally occurring DNA molecule derived from at least one template DNA molecule. The shuffled DNA preferably encodes a variant polypeptide modified with respect to the polypeptide encoded by the template DNA, and may have an altered biological activity with respect to the polypeptide encoded by the template DNA.

55 **[0099]** "Recombinant DNA molecule" is a combination of DNA sequences that are joined together using recombinant DNA technology and procedures used to join together DNA sequences as described, for example, in Sambrook et al., 1989.

[0100] The word "plant" refers to any plant, particularly to agronomically useful plants (e.g., seed plants), and "plant

cell" is a structural and physiological unit of the plant, which comprises a cell wall but may also refer to a protoplast. The plant cell may be in form of an isolated single cell or a cultured cell, or as a part of higher organized unit such as, for example, a plant tissue, or a plant organ differentiated into a structure that is present at any stage of a plant's development. Such structures include one or more plant organs including, but are not limited to, fruit, shoot, stem, leaf, flower petal, etc. Preferably, the term "plant" includes whole plants, shoot vegetative organs/structures (e.g. leaves, stems and tubers), roots, flowers and floral organs/structures (e.g. bracts, sepals, petals, stamens, carpels, anthers and ovules), seeds (including embryo, endosperm, and seed coat) and fruits (the mature ovary), plant tissues (e.g. vascular tissue, ground tissue, and the like) and cells (e.g. guard cells, egg cells, trichomes and the like), and progeny of same.

[0101] The class of plants that can be used in the method of the invention is generally as broad as the class of higher and lower plants amenable to transformation techniques, including angiosperms (monocotyledonous and dicotyledonous plants), gymnosperms, ferns, and multicellular algae. It includes plants of a variety of ploidy levels, including aneuploid, polyploid, diploid, haploid and hemizygous. Included within the scope of the invention are all genera and species of higher and lower plants of the plant kingdom. Included are furthermore the mature plants, seed, shoots and seedlings, and parts, propagation material (for example seeds and fruit) and cultures, for example cell cultures, derived therefrom. Preferred are plants and plant materials of the following plant families: *Amaranthaceae*, *Brassicaceae*, *Carophyllaceae*, *Chenopodiaceae*, *Compositae*, *Cucurbitaceae*, *Labiatae*, *Leguminosae*, *Papilionoideae*, *Liliaceae*, *Linaceae*, *Malvaceae*, *Rosaceae*, *Saxifragaceae*, *Scrophulariaceae*, *Solanaceae*, *Tetragoniaceae*.

[0102] Annual, perennial, monocotyledonous and dicotyledonous plants are preferred host organisms for the generation of transgenic plants. The use of the recombination system, or method according to the invention is furthermore advantageous in all ornamental plants, forestry, fruit, or ornamental trees, flowers, cut flowers, shrubs or turf. Said plant may include - but shall not be limited to - bryophytes such as, for example, Hepaticae (hepaticas) and Musci (mosses); pteridophytes such as ferns, horsetail and clubmosses; gymnosperms such as conifers, cycads, ginkgo and *Gnetaceae*; algae such as *Chlorophyceae*, *Phaeophyceae*, *Rhodophyceae*, *Myxophyceae*, *Xanthophyceae*, *Bacillariophyceae* (diatoms) and *Euglenophyceae*.

[0103] Plants for the purposes of the invention may comprise the families of the Rosaceae such as rose, Ericaceae such as rhododendrons and azaleas, *Euphorbiaceae* such as poinsettias and croton, *Caryophyllaceae* such as pinks, Solanaceae such as petunias, *Gesneriaceae* such as African violet, *Balsaminaceae* such as touch-me-not, Orchidaceae such as orchids, *Iridaceae* such as gladioli, iris, freesia and crocus, *Compositae* such as marigold, *Geraniaceae* such as geraniums, *Liliaceae* such as *Drachaena*, *Moraceae* such as ficus, Araceae such as philodendron and many others.

[0104] The transgenic plants according to the invention are furthermore selected in particular from among dicotyledonous crop plants such as, for example, from the families of the Leguminosae such as pea, alfalfa and soybean; the family of the *Umbelliferae*, particularly the genus *Daucus* (very particularly the species *carota* (carrot)) and *Apium* (very particularly the species *graveolens* var. dulce (celery)) and many others; the family of the Solanaceae, particularly the genus *Lycopersicon*, very particularly the species *esulentum* (tomato) and the genus *Solanum*, very particularly the species *tuberosum* (potato) and *melongena* (aubergine), tobacco and many others; and the genus *Capsicum*, very particularly the species *annum* (pepper) and many others; the family of the *Leguminosae*, particularly the genus *Glycine*, very particularly the species *max* (soybean) and many others; and the family of the *Cruciferae*, particularly the genus *Brassica*, very particularly the species *napus* (oilseed rape), *campestris* (beet), *oleracea* cv Tastié (cabbage), *oleracea* cv Snowball Y (cauliflower) and *oleracea* cv Emperor (broccoli); and the genus *Arabidopsis*, very particularly the species *thaliana* and many others; the family of the Compositae, particularly the genus *Lactuca*, very particularly the species *sativa* (lettuce) and many others.

[0105] The transgenic plants according to the invention may be selected among monocotyledonous crop plants, such as, for example, cereals such as wheat, barley, sorghum and millet, rye, triticale, maize, rice or oats, and sugarcane. Further preferred are trees such as apple, pear, quince, plum, cherry, peach, nectarine, apricot, papaya, mango, and other woody species including coniferous and deciduous trees such as poplar, pine, sequoia, cedar, oak, etc. Especially preferred are *Arabidopsis thaliana*, *Nicotiana tabacum*, oilseed rape, soybean, corn (maize), wheat, linseed, potato and Tagetes.

[0106] "Significant increase" is an increase that is larger than the margin of error inherent in the measurement technique, preferably an increase by about 2-fold or greater.

[0107] "Significantly less" means that the decrease is larger than the margin of error inherent in the measurement technique, preferably a decrease by about 2-fold or greater.

DETAILED DESCRIPTION OF THE INVENTION

[0108] The present invention thus provides for isolated nucleic acid molecules comprising a plant nucleotide sequence that directs guard cell-preferential or guard cell-specific transcription of an operably linked nucleic acid fragment in a plant cell.

[0109] Accordingly, a first embodiment of the invention relates to a expression cassette for guard cell-specific or guard

cell-preferential transcription of an operatively linked nucleic acid sequences in plants comprising

i) at least one transcription regulating nucleotide sequence selected from the group consisting of

5 a) a transcription regulating nucleotide sequence of the plant gene described by the *Arabidopsis thaliana* genome locus At5g58580, and

b) the transcription regulating nucleotide sequences as described by SEQ ID NO: 1, 2, 3, 4, 5, 6, 7, 8, 9, 12, 13, 14 and 15, the functional equivalents thereof and the functional equivalent fragments thereof,

10 and functionally linked thereto

ii) at least one nucleic acid sequence which is heterologous in relation to said transcription regulating nucleotide sequence.

15 **[0110]** The guard cell-preferential or guard cell-specific or guard cell-preferential transcription regulating nucleotide sequences of the invention may be used to manipulate the response to environmental and internal cues (e.g. light, drought, plant hormones as e.g. abscisic acid), which are finally transduced into stomatal opening or closing. It is known that various receptors and ion channel proteins play prominent roles in the signal transduction pathway. Enzymatic reactions, e.g. providing organic anions, and ion pumping into or out of the guard cell's vacuoles cause changes in cell turgor and by this influence the width of stomatal opening. Since signal transduction elements as well as enzymatic components also found in other plant cells are involved in these processes it is highly desirable to have guard cell-specific promoters at hand if one intends to manipulate metabolism or signal chains exclusively in these cells.

20 **[0111]** Possible applications of the guard cell-specific or guard cell-preferential transcription regulating nucleotide sequences of the invention might be to use them in driving expression of genes coding for enzymes or proteinaceous components of the signal transduction pathways involved in stomata opening and closing, aiming at better water use efficiency of crop plants, i.e. providing crop plants which use the available water more efficiently for their growth and development. It could also be advantageous to engineer plants in which the stomata are kept open for longer time periods, e.g. to enable plants to take up and store more carbon dioxide. By alternative approaches it could also be possible to cause stomata to close earlier and by this prevent water loss under detrimental environmental conditions, i.e. transpiration would be limited by these approaches. Another field of application in plant protection would be to use guard cell-specific promoters for regulating the expression of effect genes that finally prevent e.g. fungal pathogens from entering the plant through stomata.

25 **[0112]** The term "guard cell" in the context of the inventions means the specialized epidermal cells that regulate the aperture (i.e. the opening and closing) of stomata and by this controls the bulk of gas exchange as well as transpiration. These pairs of bean-like shaped cells are characterized by their highly regulated turgor (i.e. pressure-dependent shape), which causes the stomata to close or to open at states of low or high turgor, respectively. Guard cells derive from epidermal cells and are evenly spaced in the epidermis, i.e. the outermost cell layer of plant organs. Guard cells differ from their surrounding epidermal cells not only by shape but also by their ability to photosynthesize.

30 **[0113]** "Guard cell-specific transcription" in the context of this invention means the transcription of a nucleic acid sequence by a transcription regulating element in a way that transcription of said nucleic acid sequence in guard-cells contribute to more than 90%, preferably more than 95%, more preferably more than 99% of the entire quantity of the RNA transcribed from said nucleic acid sequence in the entire plant during any of its developmental stage. The transcription regulating element designated pSUH305S and its derivatives (pSUH305, pSUH305GB) are considered to be guard cell-specific.

35 **[0114]** "Guard cell-preferential transcription" in the context of this invention means the transcription of a nucleic acid sequence by a transcription regulating element in a way that transcription of said nucleic acid sequence in guard-cells contribute to more than 50%, preferably more than 70%, more preferably more than 80% of the entire quantity of the RNA transcribed from said nucleic acid sequence in the entire plant during any of its developmental stage. The transcription regulating element designated pSUK132, pSUK134, pSUK136, pSUK342, pSUK344 and their respective derivatives (pSUK132GB, pSUK134GB, pSUK136GB, pSUK342GB, pSUK344GB) are considered to be guard cell-preferential.

40 **[0115]** Preferably a transcription regulating nucleotide sequence of the invention comprises at least one promoter sequence of the respective gene (e.g., a sequence localized upstream of the transcription start of the respective gene capable to induce transcription of the downstream sequences). The transcription regulating nucleotide sequence may comprise the promoter sequence of said genes but may further comprise other elements such as the 5'-untranslated sequence, enhancer, introns etc. Preferably, said promoter sequence directs guard cell-preferential or guard cell-specific transcription of an operably linked nucleic acid segment in a plant or plant cell e.g., a linked plant DNA comprising an open reading frame for a structural or regulatory gene.

45 **[0116]** The following Table 1 illustrates the genes from which the promoters of the invention are preferably isolated,

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the function of said genes, the cDNA encoded by said genes, and the protein (ORF) encoded by said genes.

Table 1: Genes from which the promoters of the invention are preferably isolated, putative function of said genes, cDNA and the protein encoded by said genes.

Gene Locus	Putative function	Promoter SEQ ID	mRNA locus ID cDNA SEQ ID	Protein ID Protein SEQ ID
-	Arabidopsis thaliana genomic DNA	SEQ ID NO: 1,2, 3		
At5g58580	zinc finger (C3HC4-type RING finger) family protein	SEQ ID NO: 4,5,6,7,8,9	NM_125245 SEQ ID NO: 10	NP_200666.1 SEQ ID NO: 11
-	Arabidopsis thaliana genomic DNA	SEQ ID NO: 12,13,14,15		

[0117] While the transcription regulating elements as described by SEQ ID NO: 4, 5, 6, 7, 8, and 9 have a localization in the Arabidopsis thaliana genome suggesting a transcription regulating function also in their natural position (however giving no hint for their tissue specificity), the transcription regulating nucleotide sequences as described by SEQ ID NO: 1, 2, and 3 and SEQ ID NO: 12, 13, 14 and 15, respectively, seem to have no obvious transcription regulating function at their natural position in the genome. These sequences in their natural localization are located in no position to a coding sequence, which may suggest an operable linkage. It is especially surprising that such "orphan" sequences have a transcription regulating capability with very high tissue specificity.

[0118] Preferably, the transcription regulating nucleotide sequence (or the functional equivalent thereof) is selected from the group of sequences consisting of

i) the sequences described by SEQ ID NOs: 1, 2, 3, 4, 5, 6, 7, 8, 9, 12, 13, 14 and 15,

ii) a fragment of at least 50 consecutive bases of a sequence under i) which has substantially the same promoter activity as the corresponding transcription regulating nucleotide sequence described by SEQ ID NO: 1, 2, 3, 4, 5, 6, 7, 8, 9, 12, 13, 14 or 15;

iii) a nucleotide sequence having substantial similarity (e.g., with a sequence identity of at least 40, 50, 60, to 70%, e.g., preferably 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, to 79%, generally at least 80%, e.g., 81% to 84%, at least 85%, e.g., 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, to 98% and 99%) to a transcription regulating nucleotide sequence described by SEQ ID NO: 1, 2, 3, 4, 5, 6, 7, 8, 9, 12, 13, 14 or 15;

iv) a nucleotide sequence capable of hybridizing (preferably under conditions equivalent to hybridization in 7% sodium dodecyl sulfate (SDS), 0.5 M NaPO₄, 1 mM EDTA at 50°C with washing in 2 X SSC, 0.1% SDS at 50°C (more desirably in 7% sodium dodecyl sulfate (SDS), 0.5 M NaPO₄, 1 mM EDTA at 50°C with washing in 1 X SSC, 0.1% SDS at 50°C, more desirably still in 7% sodium dodecyl sulfate (SDS), 0.5 M NaPO₄, 1 mM EDTA at 50°C with washing in 0.5 X SSC, 0.1% SDS at 50°C, preferably in 7% sodium dodecyl sulfate (SDS), 0.5 M NaPO₄, 1 mM EDTA at 50°C with washing in 0.1 X SSC, 0.1% SDS at 50°C, more preferably in 7% sodium dodecyl sulfate (SDS), 0.5 M NaPO₄, 1 mM EDTA at 50°C with washing in 0.1 X SSC, 0.1% SDS at 65°C) to a transcription regulating nucleotide sequence described by SEQ ID NO: 1, 2, 3, 4, 5, 6, 7, 8, 9, 12, 13, 14 or 15, or the complement thereof;

v) a nucleotide sequence capable of hybridizing (preferably under conditions equivalent to hybridization in 7% sodium dodecyl sulfate (SDS), 0.5 M NaPO₄, 1 mM EDTA at 50°C with washing in 2 X SSC, 0.1% SDS at 50°C (more desirably in 7% sodium dodecyl sulfate (SDS), 0.5 M NaPO₄, 1 mM EDTA at 50°C with washing in 1 X SSC, 0.1% SDS at 50°C, more desirably still in 7% sodium dodecyl sulfate (SDS), 0.5 M NaPO₄, 1 mM EDTA at 50°C with washing in 0.5 X SSC, 0.1% SDS at 50°C, preferably in 7% sodium dodecyl sulfate (SDS), 0.5 M NaPO₄, 1 mM EDTA at 50°C with washing in 0.1 X SSC, 0.1% SDS at 50°C, more preferably in 7% sodium dodecyl sulfate (SDS), 0.5 M NaPO₄, 1 mM EDTA at 50°C with washing in 0.1 X SSC, 0.1% SDS at 65°C) to a nucleic acid comprising 50 to 200 or more consecutive nucleotides of a transcription regulating nucleotide sequence described by SEQ ID NO:

1, 2, 3, 4, 5, 6, 7, 8, 9, 12, 13, 14 or 15, or the complement thereof;

vi) a nucleotide sequence which is the complement or reverse complement of any of the previously mentioned nucleotide sequences under i) to v).

5 **[0119]** A functional equivalent of the transcription regulating nucleotide sequence can also be obtained or is obtainable from plant genomic DNA from a gene encoding a polypeptide which is substantially similar and preferably has at least 70%, preferably 80%, more preferably 90%, most preferably 95% amino acid sequence identity to a polypeptide encoded by an *Arabidopsis thaliana* gene comprising any one of SEQ ID NOs: 11, or a fragment of said transcription regulating nucleotide sequence which exhibits promoter activity in a guard cell-preferential or guard cell-specific fashion.

10 **[0120]** The activity of a transcription regulating nucleotide sequence is considered equivalent if transcription is initiated in a guard cell-preferential or guard cell-specific fashion (as defined above). Such expression profile is preferably demonstrated using reporter genes operably linked to said transcription regulating nucleotide sequence. Preferred reporter genes (Schenborn 1999) in this context are green fluorescence protein (GFP) (Chui 1996; Leffel 1997), chloramphenicol transferase, luciferase (Millar 1992), β -glucuronidase or β -galactosidase. Especially preferred is β -glucuronidase (Jefferson 1987).

15 **[0121]** Beside this the transcription regulating activity of a function equivalent may vary from the activity of its parent sequence, especially with respect to expression level. The expression level may be higher or lower than the expression level of the parent sequence. Both derivations may be advantageous depending on the nucleic acid sequence of interest to be expressed. Preferred are such functional equivalent sequences which - in comparison with its parent sequence - does not deviate from the expression level of said parent sequence by more than 50%, preferably 25%, more preferably 10% (as to be preferably judged by either mRNA expression or protein (e.g., reporter gene) expression). Furthermore preferred are equivalent sequences which demonstrate an increased expression in comparison to its parent sequence, preferably an increase by at least 50%, more preferably by at least 100%, most preferably by at least 500%.

20 **[0122]** Preferably functional equivalent of the transcription regulating nucleotide sequence can be obtained or is obtainable from plant genomic DNA from a gene expressing a mRNA described by a cDNA which is substantially similar and preferably has at least 70%, preferably 80%, more preferably 90%, most preferably 95% sequence identity to a sequence described by any one of SEQ ID NOs: 10, or a fragment of said transcription regulating nucleotide sequence which exhibits promoter activity in a guard cell-preferential or guard cell-specific fashion.

25 **[0123]** Such functional equivalent of the transcription regulating nucleotide sequence may be obtained from other plant species by using the guard cell-preferential or guard cell-specific *Arabidopsis* promoter sequences described herein as probes to screen for homologous structural genes in other plants by hybridization under low, moderate or stringent hybridization conditions. Regions of the guard cell-preferential or guard cell-specific promoter sequences of the present invention which are conserved among species could also be used as PCR primers to amplify a segment from a species other than *Arabidopsis*, and that segment used as a hybridization probe (the latter approach permitting higher stringency screening) or in a transcription assay to determine promoter activity. Moreover, the guard cell-preferential or guard cell-specific promoter sequences could be employed to identify structurally related sequences in a database using computer algorithms.

30 **[0124]** More specifically, based on the *Arabidopsis* nucleic acid sequences of the present invention, orthologs may be identified or isolated from the genome of any desired organism, preferably from another plant, according to well known techniques based on their sequence similarity to the *Arabidopsis* nucleic acid sequences, e.g., hybridization, PCR or computer generated sequence comparisons. For example, all or a portion of a particular *Arabidopsis* nucleic acid sequence is used as a probe that selectively hybridizes to other gene sequences present in a population of cloned genomic DNA fragments or cDNA fragments (i.e., genomic or cDNA libraries) from a chosen source organism. Further, suitable genomic and cDNA libraries may be prepared from any cell or tissue of an organism. Such techniques include hybridization screening of plated DNA libraries (either plaques or colonies; see, e.g., Sambrook 1989) and amplification by PCR using oligonucleotide primers preferably corresponding to sequence domains conserved among related polypeptide or subsequences of the nucleotide sequences provided herein (see, e.g., Innis 1990). These methods are particularly well suited to the isolation of gene sequences from organisms closely related to the organism from which the probe sequence is derived. The application of these methods using the *Arabidopsis* sequences as probes is well suited for the isolation of gene sequences from any source organism, preferably other plant species. In a PCR approach, oligonucleotide primers can be designed for use in PCR reactions to amplify corresponding DNA sequences from cDNA or genomic DNA extracted from any plant of interest. Methods for designing PCR primers and PCR cloning are generally known in the art.

35 **[0125]** In hybridization techniques, all or part of a known nucleotide sequence is used as a probe that selectively hybridizes to other corresponding nucleotide sequences present in a population of cloned genomic DNA fragments or cDNA fragments (i.e., genomic or cDNA libraries) from a chosen organism. The hybridization probes may be genomic DNA fragments, cDNA fragments, RNA fragments, or other oligonucleotides, and may be labeled with a detectable

group such as ³²P, or any other detectable marker. Thus, for example, probes for hybridization can be made by labeling synthetic oligonucleotides based on the sequence of the invention. Methods for preparation of probes for hybridization and for construction of cDNA and genomic libraries are generally known in the art and are disclosed in Sambrook et al. (1989). In general, sequences that hybridize to the sequences disclosed herein will have at least 40% to 50%, about 60% to 70% and even about 80% 85%, 90%, 95% to 98% or more identity with the disclosed sequences. That is, the sequence similarity of sequences may range, sharing at least about 40% to 50%, about 60% to 70%, and even about 80%, 85%, 90%, 95% to 98% sequence similarity.

[0126] The nucleic acid molecules of the invention can also be identified by, for example, a search of known databases for genes encoding polypeptides having a specified amino acid sequence identity or DNA having a specified nucleotide sequence identity. Methods of alignment of sequences for comparison are well known in the art and are described hereinabove.

[0127] Hence, the isolated nucleic acid molecules of the invention include the orthologs of the *Arabidopsis* sequences disclosed herein, i.e., the corresponding nucleotide sequences in organisms other than *Arabidopsis*, including, but not limited to, plants other than *Arabidopsis*, preferably dicotyledonous plants, e.g., Brassica napus, alfalfa, sunflower, soybean, cotton, peanut, tobacco or sugar beet, but also cereal plants such as corn, wheat, rye, turfgrass, sorghum, millet, sugarcane, barley and banana. An orthologous gene is a gene from a different species that encodes a product having the same or similar function, e.g., catalyzing the same reaction as a product encoded by a gene from a reference organism. Thus, an ortholog includes polypeptides having less than, e.g., 65% amino acid sequence identity, but which ortholog encodes a polypeptide having the same or similar function. Databases such GenBank may be employed to identify sequences related to the *Arabidopsis* sequences, e.g., orthologs in other dicotyledonous plants such as Brassica napus and others. Alternatively, recombinant DNA techniques such as hybridization or PCR may be employed to identify sequences related to the *Arabidopsis* sequences or to clone the equivalent sequences from different *Arabidopsis* DNAs.

[0128] The transcription regulating nucleotide sequences of the invention or their functional equivalents can be obtained or isolated from any plant or non-plant source, or produced synthetically by purely chemical means. Preferred sources include, but are not limited to the plants defined in the DEFINITION section above.

[0129] Thus, another embodiment of the invention relates to a method for identifying and/or isolating a sequence with guard cell-preferential or guard cell-specific transcription regulating activity utilizing a nucleic acid sequence encoding a amino acid sequence as described by SEQ ID NO: 11 or a part thereof. Preferred are nucleic acid sequences described by SEQ ID NO: 10 or parts thereof. "Part" in this context means a nucleic acid sequence of at least 15 bases preferably at least 25 bases, more preferably at least 50 bases. The method can be based on (but is not limited to) the methods described above such as polymerase chain reaction, hybridization or database screening. Preferably, this method of the invention is based on a polymerase chain reaction, wherein said nucleic acid sequence or its part is utilized as oligonucleotide primer. The person skilled in the art is aware of several methods to amplify and isolate the promoter of a gene starting from part of its coding sequence (such as, for example, part of a cDNA). Such methods may include but are not limited to method such as inverse PCR ("iPCR") or "thermal asymmetric interlaced PCR" ("TAIL PCR").

[0130] Another embodiment of the invention is related to a method for providing a transgenic expression cassette for guard cell-preferential or guard cell-specific expression comprising the steps of:

I. isolating of a guard cell-preferential or guard cell-specific transcription regulating nucleotide sequence utilizing at least one nucleic acid sequence or a part thereof, wherein said sequence is encoding a polypeptide described by SEQ ID NO: 11, or a part of at least 15 bases thereof, and

II. functionally linking said guard cell-preferential or guard cell-specific transcription regulating nucleotide sequence to another nucleotide sequence of interest, which is heterologous in relation to said guard cell-preferential or guard cell-specific transcription regulating nucleotide sequence.

[0131] Preferably, the nucleic acid sequence employed for the isolation comprises at least 15 base, preferably at least 25 bases, more preferably at least 50 bases of a sequence described by SEQ ID NO: 10. Preferably, the isolation of the guard cell-preferential or guard cell-specific transcription regulating nucleotide sequence is realized by a polymerase chain reaction utilizing said nucleic acid sequence as a primer. The operable linkage can be realized by standard cloning method known in the art such as ligation-mediated cloning or recombination-mediated cloning.

[0132] Preferably, the transcription regulating nucleotide sequences and promoters of the invention include a consecutive stretch of about 25 to 2000, including 50 to 500 or 100 to 250, and up to 1000 or 1500, contiguous nucleotides, e.g., 40 to about 743, 60 to about 743, 125 to about 743, 250 to about 743, 400 to about 743, 600 to about 743, of any one of SEQ ID NOs: 1, 2, 3, 4, 5, 6, 7, 8, 9, 12, 13, 14, and 15, or the promoter orthologs thereof, which include the minimal promoter region.

[0133] In a particular embodiment of the invention said consecutive stretch of about 25 to 2000, including 50 to 500 or 100 to 250, and up to 1000 or 1500, contiguous nucleotides, e.g., 40 to about 743, 60 to about 743, 125 to about 743,

250 to about 743, 400 to about 743, 600 to about 743, has at least 75%, preferably 80%, more preferably 90% and most preferably 95%, nucleic acid sequence identity with a corresponding consecutive stretch of about 25 to 2000, including 50 to 500 or 100 to 250, and up to 1000 or 1500, contiguous nucleotides, e.g., 40 to about 743, 60 to about 743, 125 to about 743, 250 to about 743, 400 to about 743, 600 to about 743, of any one of SEQ ID NOs: 1, 2, 3, 4, 5, 6, 7, 8, 9, 12, 13, 14, and 15, or the promoter orthologs thereof, which include the minimal promoter region. The above defined stretch of contiguous nucleotides preferably comprises one or more promoter motifs selected from the group consisting of TATA box, GC-box, CAAT-box and a transcription start site.

[0134] The transcription regulating nucleotide sequences of the invention or their functional equivalents are capable of driving guard cell-preferential or guard cell-specific expression of a coding sequence in a target cell, particularly in a plant cell. The promoter sequences and methods disclosed herein are useful in regulating guard cell-preferential or guard cell-specific expression, respectively, of any heterologous nucleotide sequence in a host plant in order to vary the phenotype of that plant. These promoters can be used with combinations of enhancer, upstream elements, and/or activating sequences from the 5' flanking regions of plant expressible structural genes. Similarly the upstream element can be used in combination with various plant promoter sequences.

[0135] The transcription regulating nucleotide sequences and promoters of the invention are useful to modify the phenotype of a plant. Various changes in the phenotype of a transgenic plant are desirable, i.e., modifying the fatty acid composition in a plant, altering the amino acid content of a plant, altering a plant's pathogen defense mechanism, and the like. These results can be achieved by providing expression of heterologous products or increased expression of endogenous products in plants. Alternatively, the results can be achieved by providing for a reduction of expression of one or more endogenous products, particularly enzymes or cofactors in the plant. These changes result in an alteration in the phenotype of the transformed plant.

[0136] Generally, the transcription regulating nucleotide sequences and promoters of the invention may be employed to express a nucleic acid segment that is operably linked to said promoter such as, for example, an open reading frame, or a portion thereof, an anti-sense sequence, a sequence encoding for a double-stranded RNA sequence, or a transgene in plants.

[0137] An operable linkage may - for example - comprise an sequential arrangement of the transcription regulating nucleotide sequence of the invention (for example a sequence as described by SEQ ID NO: 1, 2, 3, 4, 5, 6, 7, 8, 9, 12, 13, 14 or 15) with a nucleic acid sequence to be expressed, and - optionally - additional regulatory elements such as for example polyadenylation or transcription termination elements, enhancers, introns etc, in a way that the transcription regulating nucleotide sequence can fulfill its function in the process of expression the nucleic acid sequence of interest under the appropriate conditions. the term "appropriate conditions" mean preferably the presence of the expression cassette in a plant cell. Preferred are arrangements, in which the nucleic acid sequence of interest to be expressed is placed down-stream (i.e., in 3'-direction) of the transcription regulating nucleotide sequence of the invention in a way, that both sequences are covalently linked. Optionally additional sequences may be inserted in-between the two sequences. Such sequences may be for example linker or multiple cloning sites. Furthermore, sequences can be inserted coding for parts of fusion proteins (in case a fusion protein of the protein encoded by the nucleic acid of interest is intended to be expressed). Preferably, the distance between the nucleic acid sequence of interest to be expressed and the transcription regulating nucleotide sequence of the invention is not more than 200 base pairs, preferably not more than 100 base pairs, more preferably no more than 50 base pairs.

[0138] An operable linkage in relation to any expression cassette or of the invention may be realized by various methods known in the art, comprising both *in vitro* and *in vivo* procedure. Thus, an expression cassette of the invention or an vector comprising such expression cassette may be realized using standard recombination and cloning techniques well known in the art (see e.g., Maniatis 1989; Silhavy 1984; Ausubel 1987).

[0139] An expression cassette may also be assembled by inserting a transcription regulating nucleotide sequence of the invention (for example a sequence as described by SEQ ID NO: 1, 2, 3, 4, 5, 6, 7, 8, 9, 12, 13, 14 or 15) into the plant genome. Such insertion will result in an operable linkage to a nucleic acid sequence of interest which as such already existed in the genome. By the insertion the nucleic acid of interest is expressed in a guard cell-preferential or guard cell-specific way due to the transcription regulating properties of the transcription regulating nucleotide sequence. The insertion may be directed or by chance. Preferably the insertion is directed and realized by for example homologous recombination. By this procedure a natural promoter may be exchanged against the transcription regulating nucleotide sequence of the invention, thereby modifying the expression profile of an endogenous gene. The transcription regulating nucleotide sequence may also be inserted in a way, that antisense mRNA of an endogenous gene is expressed, thereby inducing gene silencing.

[0140] Similar, a nucleic acid sequence of interest to be expressed may be inserted into a plant genome comprising the transcription regulating nucleotide sequence in its natural genomic environment (i.e. linked to its natural gene) in a way that the inserted sequence becomes operably linked to the transcription regulating nucleotide sequence, thereby forming an expression cassette of the invention.

[0141] Most preferably a guard cell-specific or guard cell-preferential transcription regulating nucleotide sequence of

the invention is used to express traits which are especially of use for specific expression in guard-cells. Examples for such traits are given above.

[0142] However, principally many traits may be useful to be expressed in guard cells. The open reading frame to be linked to the transcription regulating nucleotide sequence of the invention may be obtained from an insect resistance gene, a disease resistance gene such as, for example, a bacterial disease resistance gene, a fungal disease resistance gene, a viral disease resistance gene, a nematode disease resistance gene, a herbicide resistance gene, a gene affecting grain composition or quality, a nutrient utilization gene, a mycotoxin reduction gene, a male sterility gene, a selectable marker gene, a screenable marker gene, a negative selectable marker, a positive selectable marker, a gene affecting plant agronomic characteristics, i.e., yield, standability, and the like, or an environment or stress resistance gene, i.e., one or more genes that confer herbicide resistance or tolerance, insect resistance or tolerance, disease resistance or tolerance (viral, bacterial, fungal, oomycete, or nematode), stress tolerance or resistance (as exemplified by resistance or tolerance to drought, heat, chilling, freezing, excessive moisture, salt stress, or oxidative stress), increased yields, food content and makeup, physical appearance, male sterility, drydown, standability, prolificacy, starch properties or quantity, oil quantity and quality, amino acid or protein composition, and the like. By "resistant" is meant a plant which exhibits substantially no phenotypic changes as a consequence of agent administration, infection with a pathogen, or exposure to stress. By "tolerant" is meant a plant which, although it may exhibit some phenotypic changes as a consequence of infection, does not have a substantially decreased reproductive capacity or substantially altered metabolism.

[0143] Guard cell-preferential or guard cell-specific transcription regulating nucleotide sequences (e.g., promoters) are useful for expressing a wide variety of genes including those which alter metabolic pathways, confer disease resistance, for protein production, e.g., antibody production, or to improve nutrient uptake and the like. Guard cell-preferential or guard cell-specific transcription regulating nucleotide sequences (e.g., promoters) may be modified so as to be regulatable, e.g., inducible. The genes and transcription regulating nucleotide sequences (e.g., promoters) described hereinabove can be used to identify orthologous genes and their transcription regulating nucleotide sequences (e.g., promoters) which are also likely expressed in a particular tissue and/or development manner. Moreover, the orthologous transcription regulating nucleotide sequences (e.g., promoters) are useful to express linked open reading frames. In addition, by aligning the transcription regulating nucleotide sequences (e.g., promoters) of these orthologs, novel cis elements can be identified that are useful to generate synthetic transcription regulating nucleotide sequences (e.g., promoters).

[0144] The expression regulating nucleotide sequences specified above may be optionally operably linked to other suitable regulatory sequences, e.g., a transcription terminator sequence, operator, repressor binding site, transcription factor binding site and/or an enhancer.

[0145] The present invention further provides a recombinant vector containing the expression cassette of the invention, and host cells comprising the expression cassette or vector, e.g., comprising a plasmid. The expression cassette or vector may augment the genome of a transformed plant or may be maintained extra chromosomally. The expression cassette or vector of the invention may be present in the nucleus, chloroplast, mitochondria and/or plastid of the cells of the plant. Preferably, the expression cassette or vector of the invention is comprised in the chromosomal DNA of the plant nucleus. The present invention also provides a transgenic plant prepared by this method, a seed from such a plant and progeny plants from such a plant including hybrids and inbreds. The expression cassette may be operatively linked to a structural gene, the open reading frame thereof, or a portion thereof. The expression cassette may further comprise a Ti plasmid and be contained in an *Agrobacterium tumefaciens* cell; it may be carried on a microparticle, wherein the microparticle is suitable for ballistic transformation of a plant cell; or it may be contained in a plant cell or protoplast. Further, the expression cassette or vector can be contained in a transformed plant or cells thereof, and the plant may be a dicot or a monocot. In particular, the plant may be a dicotyledonous plant. Preferred transgenic plants are transgenic maize, soybean, barley, alfalfa, sunflower, canola, soybean, cotton, peanut, sorghum, tobacco, sugarbeet, rice, wheat, rye, turfgrass, millet, sugarcane, tomato, or potato.

[0146] The invention also provides a method of plant breeding, e.g., to prepare a crossed fertile transgenic plant. The method comprises crossing a fertile transgenic plant comprising a particular expression cassette of the invention with itself or with a second plant, e.g., one lacking the particular expression cassette, to prepare the seed of a crossed fertile transgenic plant comprising the particular expression cassette. The seed is then planted to obtain a crossed fertile transgenic plant. The plant may be a monocot or a dicot. In a particular embodiment, the plant is a dicotyledonous plant. The crossed fertile transgenic plant may have the particular expression cassette inherited through a female parent or through a male parent. The second plant may be an inbred plant. The crossed fertile transgenic may be a hybrid. Also included within the present invention are seeds of any of these crossed fertile transgenic plants.

[0147] The transcription regulating nucleotide sequences of the invention further comprise sequences which are complementary to one (hereinafter "test" sequence) which hybridizes under stringent conditions with a nucleic acid molecule as described by SEQ ID NO: 1, 2, 3, 4, 5, 6, 7, 8, 9, 12, 13, 14, or 15 as well as RNA which is transcribed from the nucleic acid molecule. When the hybridization is performed under stringent conditions, either the test or nucleic acid molecule of invention is preferably supported, e.g., on a membrane or DNA chip. Thus, either a denatured test or nucleic

acid molecule of the invention is preferably first bound to a support and hybridization is effected for a specified period of time at a temperature of, e.g., between 55°C and 70°C, in double strength citrate buffered saline (SC) containing 0.1 % SDS followed by rinsing of the support at the same temperature but with a buffer having a reduced SC concentration. Depending upon the degree of stringency required such reduced concentration buffers are typically single strength SC containing 0.1 % SDS, half strength SC containing 0.1% SDS and one-tenth strength SC containing 0.1 % SDS. More preferably hybridization is carried out under high stringency conditions (as defined above).

[0148] Virtually any DNA composition may be used for delivery to recipient plant cells, e.g., dicotyledonous cells, to ultimately produce fertile transgenic plants in accordance with the present invention. For example, DNA segments or fragments in the form of vectors and plasmids, or linear DNA segments or fragments, in some instances containing only the DNA element to be expressed in the plant, and the like, may be employed. The construction of vectors which may be employed in conjunction with the present invention will be known to those of skill of the art in light of the present disclosure (see, e.g., Sambrook 1989; Gelvin 1990).

[0149] Vectors, plasmids, cosmids, YACs (yeast artificial chromosomes), BACs (bacterial artificial chromosomes) and DNA segments for use in transforming such cells will, of course, generally comprise the cDNA, gene or genes which one desires to introduce into the cells. These DNA constructs can further include structures such as promoters, enhancers, polylinkers, or even regulatory genes as desired. The DNA segment, fragment or gene chosen for cellular introduction will often encode a protein which will be expressed in the resultant recombinant cells, such as will result in a screenable or selectable trait and/or which will impart an improved phenotype to the regenerated plant. However, this may not always be the case, and the present invention also encompasses transgenic plants incorporating non-expressed transgenes.

[0150] In certain embodiments, it is contemplated that one may wish to employ replication-competent viral vectors in monocot transformation. Such vectors include, for example, wheat dwarf virus (WDV) "shuttle" vectors, such as pW1-11 and PW1-GUS (Ugaki 1991). These vectors are capable of autonomous replication in maize cells as well as *E. coli*, and as such may provide increased sensitivity for detecting DNA delivered to transgenic cells. A replicating vector may also be useful for delivery of genes flanked by DNA sequences from transposable elements such as *Ac*, *Ds*, or *Mu*. It has been proposed (Laufs 1990) that transposition of these elements within the maize genome requires DNA replication. It is also contemplated that transposable elements would be useful for introducing DNA segments or fragments lacking elements necessary for selection and maintenance of the plasmid vector in bacteria, e.g., antibiotic resistance genes and origins of DNA replication. It is also proposed that use of a transposable element such as *Ac*, *Ds*, or *Mu* would actively promote integration of the desired DNA and hence increase the frequency of stably transformed cells. The use of a transposable element such as *Ac*, *Ds*, or *Mu* may actively promote integration of the DNA of interest and hence increase the frequency of stably transformed cells. Transposable elements may be useful to allow separation of genes of interest from elements necessary for selection and maintenance of a plasmid vector in bacteria or selection of a transformant. By use of a transposable element, desirable and undesirable DNA sequences may be transposed apart from each other in the genome, such that through genetic segregation in progeny, one may identify plants with either the desirable undesirable DNA sequences.

[0151] The nucleotide sequence of interest linked to one or more of the transcription regulating nucleotide sequences of the invention can, for example, code for a ribosomal RNA, an antisense RNA or any other type of RNA that is not translated into protein. In another preferred embodiment of the invention, said nucleotide sequence of interest is translated into a protein product. The transcription regulating nucleotide sequence and/or nucleotide sequence of interest linked thereto may be of homologous or heterologous origin with respect to the plant to be transformed. A recombinant DNA molecule useful for introduction into plant cells includes that which has been derived or isolated from any source, that may be subsequently characterized as to structure, size and/or function, chemically altered, and later introduced into plants. An example of a nucleotide sequence or segment of interest "derived" from a source, would be a nucleotide sequence or segment that is identified as a useful fragment within a given organism, and which is then chemically synthesized in essentially pure form. An example of such a nucleotide sequence or segment of interest "isolated" from a source, would be nucleotide sequence or segment that is excised or removed from said source by chemical means, e.g., by the use of restriction endonucleases, so that it can be further manipulated, e.g., amplified, for use in the invention, by the methodology of genetic engineering. Such a nucleotide sequence or segment is commonly referred to as "recombinant."

[0152] Therefore a useful nucleotide sequence, segment or fragment of interest includes completely synthetic DNA, semi-synthetic DNA, DNA isolated from biological sources, and DNA derived from introduced RNA. Generally, the introduced DNA is not originally resident in the plant genotype which is the recipient of the DNA, but it is within the scope of the invention to isolate a gene from a given plant genotype, and to subsequently introduce multiple copies of the gene into the same genotype, e.g., to enhance production of a given gene product such as a storage protein or a protein that confers tolerance or resistance to water deficit.

[0153] The introduced recombinant DNA molecule includes but is not limited to, DNA from plant genes, and non-plant genes such as those from bacteria, yeasts, animals or viruses. The introduced DNA can include modified genes, portions of genes, or chimeric genes, including genes from the same or different genotype. The term "chimeric gene" or "chimeric

DNA" is defined as a gene or DNA sequence or segment comprising at least two DNA sequences or segments from species which do not combine DNA under natural conditions, or which DNA sequences or segments are positioned or linked in a manner which does not normally occur in the native genome of untransformed plant.

5 [0154] The introduced recombinant DNA molecule used for transformation herein may be circular or linear, double-stranded or single-stranded. Generally, the DNA is in the form of chimeric DNA, such as plasmid DNA, that can also contain coding regions flanked by regulatory sequences which promote the expression of the recombinant DNA present in the resultant plant. Generally, the introduced recombinant DNA molecule will be relatively small, i.e., less than about 30 kb to minimize any susceptibility to physical, chemical, or enzymatic degradation which is known to increase as the size of the nucleotide molecule increases. As noted above, the number of proteins, RNA transcripts or mixtures thereof which is introduced into the plant genome is preferably preselected and defined, e.g., from one to about 5-10 such products of the introduced DNA may be formed.

10 [0155] Two principal methods for the control of expression are known, viz.: overexpression and underexpression. Overexpression can be achieved by insertion of one or more than one extra copy of the selected gene. It is, however, not unknown for plants or their progeny, originally transformed with one or more than one extra copy of a nucleotide sequence, to exhibit the effects of underexpression as well as overexpression. For underexpression there are two principle methods which are commonly referred to in the art as "antisense downregulation" and "sense downregulation" (sense downregulation is also referred to as "cosuppression"). Generically these processes are referred to as "gene silencing". Both of these methods lead to an inhibition of expression of the target gene.

15 [0156] Obtaining sufficient levels of transgene expression in the appropriate plant tissues is an important aspect in the production of genetically engineered crops. Expression of heterologous DNA sequences in a plant host is dependent upon the presence of an operably linked promoter that is functional within the plant host. Choice of the promoter sequence will determine when and where within the organism the heterologous DNA sequence is expressed.

20 [0157] It is specifically contemplated by the inventors that one could mutagenize a promoter to potentially improve the utility of the elements for the expression of transgenes in plants. The mutagenesis of these elements can be carried out at random and the mutagenized promoter sequences screened for activity in a trial-by-error procedure. Alternatively, particular sequences which provide the promoter with desirable expression characteristics, or the promoter with expression enhancement activity, could be identified and these or similar sequences introduced into the sequences via mutation. It is further contemplated that one could mutagenize these sequences in order to enhance their expression of transgenes in a particular species.

25 [0158] The means for mutagenizing a DNA segment encoding a promoter sequence of the current invention are well-known to those of skill in the art. As indicated, modifications to promoter or other regulatory element may be made by random, or site-specific mutagenesis procedures. The promoter and other regulatory element may be modified by altering their structure through the addition or deletion of one or more nucleotides from the sequence which encodes the corresponding unmodified sequences.

30 [0159] Mutagenesis may be performed in accordance with any of the techniques known in the art, such as, and not limited to, synthesizing an oligonucleotide having one or more mutations within the sequence of a particular regulatory region. In particular, site-specific mutagenesis is a technique useful in the preparation of promoter mutants, through specific mutagenesis of the underlying DNA. The technique further provides a ready ability to prepare and test sequence variants, for example, incorporating one or more of the foregoing considerations, by introducing one or more nucleotide sequence changes into the DNA. Site-specific mutagenesis allows the production of mutants through the use of specific oligonucleotide sequences which encode the DNA sequence of the desired mutation, as well as a sufficient number of adjacent nucleotides, to provide a primer sequence of sufficient size and sequence complexity to form a stable duplex on both sides of the deletion junction being traversed. Typically, a primer of about 17 to about 75 nucleotides or more in length is preferred, with about 10 to about 25 or more residues on both sides of the junction of the sequence being altered.

35 [0160] In general, the technique of site-specific mutagenesis is well known in the art, as exemplified by various publications. As will be appreciated, the technique typically employs a phage vector which exists in both a single stranded and double stranded form. Typical vectors useful in site-directed mutagenesis include vectors such as the M13 phage. These phage are readily commercially available and their use is generally well known to those skilled in the art. Double stranded plasmids also are routinely employed in site directed mutagenesis which eliminates the step of transferring the gene of interest from a plasmid to a phage.

40 [0161] In general, site-directed mutagenesis in accordance herewith is performed by first obtaining a single-stranded vector or melting apart of two strands of a double stranded vector which includes within its sequence a DNA sequence which encodes the promoter. An oligonucleotide primer bearing the desired mutated sequence is prepared, generally synthetically. This primer is then annealed with the single-stranded vector, and subjected to DNA polymerizing enzymes such as E. coli polymerase I Klenow fragment, in order to complete the synthesis of the mutation-bearing strand. Thus, a heteroduplex is formed wherein one strand encodes the original non-mutated sequence and the second strand bears the desired mutation. This heteroduplex vector is then used to transform or transfect appropriate cells, such as E. coli cells, and cells are selected which include recombinant vectors bearing the mutated sequence arrangement. Vector

DNA can then be isolated from these cells and used for plant transformation. A genetic selection scheme was devised by Kunkel et al. (1987) to enrich for clones incorporating mutagenic oligonucleotides. Alternatively, the use of PCR with commercially available thermostable enzymes such as Taq polymerase may be used to incorporate a mutagenic oligonucleotide primer into an amplified DNA fragment that can then be cloned into an appropriate cloning or expression vector. The PCR-mediated mutagenesis procedures of Tomic et al. (1990) and Upender et al. (1995) provide two examples of such protocols. A PCR employing a thermostable ligase in addition to a thermostable polymerase also may be used to incorporate a phosphorylated mutagenic oligonucleotide into an amplified DNA fragment that may then be cloned into an appropriate cloning or expression vector. The mutagenesis procedure described by Michael (1994) provides an example of one such protocol.

[0162] The preparation of sequence variants of the selected promoter-encoding DNA segments using site-directed mutagenesis is provided as a means of producing potentially useful species and is not meant to be limiting as there are other ways in which sequence variants of DNA sequences may be obtained. For example, recombinant vectors encoding the desired promoter sequence may be treated with mutagenic agents, such as hydroxylamine, to obtain sequence variants.

[0163] As used herein; the term "oligonucleotide directed mutagenesis procedure" refers to template-dependent processes and vector-mediated propagation which result in an increase in the concentration of a specific nucleic acid molecule relative to its initial concentration, or in an increase in the concentration of a detectable signal, such as amplification. As used herein, the term "oligonucleotide directed mutagenesis procedure" also is intended to refer to a process that involves the template-dependent extension of a primer molecule. The term template-dependent process refers to nucleic acid synthesis of an RNA or a DNA molecule wherein the sequence of the newly synthesized strand of nucleic acid is dictated by the well-known rules of complementary base pairing (see, for example, Watson and Rarnstad, 1987). Typically, vector mediated methodologies involve the introduction of the nucleic acid fragment into a DNA or RNA vector, the clonal amplification of the vector, and the recovery of the amplified nucleic acid fragment. Examples of such methodologies are provided by U.S. Pat. No. 4,237,224. A number of template dependent processes are available to amplify the target sequences of interest present in a sample, such methods being well known in the art and specifically disclosed herein below.

[0164] Where a clone comprising a promoter has been isolated in accordance with the instant invention, one may wish to delimit the essential promoter regions within the clone. One efficient, targeted means for preparing mutagenizing promoters relies upon the identification of putative regulatory elements within the promoter sequence. This can be initiated by comparison with promoter sequences known to be expressed in similar tissue-specific or developmentally unique manner. Sequences which are shared among promoters with similar expression patterns are likely candidates for the binding of transcription factors and are thus likely elements which confer expression patterns. Confirmation of these putative regulatory elements can be achieved by deletion analysis of each putative regulatory region followed by functional analysis of each deletion construct by assay of a reporter gene which is functionally attached to each construct. As such, once a starting promoter sequence is provided, any of a number of different deletion mutants of the starting promoter could be readily prepared.

[0165] Functionally equivalent fragments of a transcription regulating nucleotide sequence of the invention can also be obtained by removing or deleting non-essential sequences without deleting the essential one. Narrowing the transcription regulating nucleotide sequence to its essential, transcription mediating elements can be realized in vitro by trial-and-arrow deletion mutations, or in silico using promoter element search routines. Regions essential for promoter activity often demonstrate clusters of certain, known promoter elements. Such analysis can be performed using available computer algorithms such as PLACE ("Plant Cis-acting Regulatory DNA Elements"; Higo 1999), the BIOBASE database "Transfac" (Biologische Datenbanken GmbH, Braunschweig; Wingender 2001) or the database PlantCARE (Lescot 2002).

[0166] Preferably, functional equivalent fragments of one of the transcription regulating nucleotide sequences of the invention comprises at least 100 base pairs, preferably, at least 200 base pairs, more preferably at least 500 base pairs of a transcription regulating nucleotide sequence as described by SEQ ID NO: 1, 2, 3, 4, 5, 6, 7, 8, 9, 12, 13, 14, or 15. More preferably this fragment is starting from the 3'-end of the indicated sequences.

[0167] Especially preferred are equivalent fragments of transcription regulating nucleotide sequences, which are obtained by deleting the region encoding the 5'-untranslated region of the mRNA, thus only providing the (untranscribed) promoter region. The 5'-untranslated region can be easily determined by methods known in the art (such as 5'-RACE analysis). Accordingly, some of the transcription regulating nucleotide sequences of the invention are equivalent fragments of other sequences (see Table 2 below).

Table 2: Relationship of transcription regulating nucleotide sequences of the invention

Transcription regulating sequence	Equivalent sequence	Equivalent fragment
SEQ ID NO: 2 (931 bp)	SEQ ID NO: 3 (933 bp)	

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(continued)

Transcription regulating sequence	Equivalent sequence	Equivalent fragment
	SEQ ID NO: 1 (1001 bp) (chimeric promoter comprising also a Ds transposon derived sequence)	
SEQ ID NO: 4 (2153 bp)	SEQ ID NO: 5 (2165 bp)	SEQ ID NO: 6 (1009 bp) SEQ ID NO: 7 (1023 bp) SEQ ID NO: 8 (956 bp) SEQ ID NO: 9 (956 bp)
SEQ ID NO: 14 (2005 bp)	SEQ ID NO: 15 (2028 bp)	SEQ ID NO: 12 (912 bp) SEQ ID NO: 13 (936 bp)

[0168] As indicated above, deletion mutants of the promoter of the invention also could be randomly prepared and then assayed. With this strategy, a series of constructs are prepared, each containing a different portion of the clone (a subclone), and these constructs are then screened for activity. A suitable means for screening for activity is to attach a deleted promoter or intron construct which contains a deleted segment to a selectable or screenable marker, and to isolate only those cells expressing the marker gene. In this way, a number of different, deleted promoter constructs are identified which still retain the desired, or even enhanced, activity. The smallest segment which is required for activity is thereby identified through comparison of the selected constructs. This segment may then be used for the construction of vectors for the expression of exogenous genes.

[0169] An expression cassette of the invention may comprise further regulatory elements. The term in this context is to be understood in the a broad meaning comprising all sequences which may influence construction or function of the expression cassette. Regulatory elements may for example modify transcription and/or translation in prokaryotic or eukaryotic organism. In an preferred embodiment the expression cassette of the invention comprised downstream (in 3'-direction) of the nucleic acid sequence to be expressed a transcription termination sequence and - optionally additional regulatory elements - each operably linked to the nucleic acid sequence to be expressed (or the transcription regulating nucleotide sequence).

[0170] Additional regulatory elements may comprise additional promoter, minimal promoters, promoter elements, or transposon elements which may modify or enhance the expression regulating properties. The transcription regulating nucleotide sequence as described by SEQ ID NO: 1 (designated pSUH305) is a chimeric sequence comprising a transcription regulating nucleotide sequence from *Arabidopsis thaliana* (as described by SEQ ID NO: 2) and a short stretch of sequence derived from the 3' terminus of the transposon Ds. For example the expression may be made depending on certain stress factors such water stress, abscisic acid (Lam 1991) or heat stress (Schoffl 1989). Furthermore additional promoters or promoter elements may be employed, which may realize expression in other organisms (such as *E. coli* or *Agrobacterium*). Such regulatory elements can be found in the promoter sequences of bacteria such as amy and SPO2 or in the promoter sequences of yeast or fungal promoters (such as ADC1, MFa, AC, P-60, CYC1, GAPDH, TEF, rp28, and ADH).

[0171] Furthermore, it is contemplated that promoters combining elements from more than one promoter may be useful. For example, US 5,491,288 discloses combining a Cauliflower Mosaic Virus promoter with a histone promoter. Thus, the elements from the promoters disclosed herein may be combined with elements from other promoters. Promoters which are useful for plant transgene expression include those that are inducible, viral, synthetic, constitutive (Odell 1985), temporally regulated, spatially regulated, tissue-specific, and spatial-temporally regulated.

[0172] Where expression in specific tissues or organs is desired, tissue-specific promoters may be used. In contrast, where gene expression in response to a stimulus is desired, inducible promoters are the regulatory elements of choice. Where continuous expression is desired throughout the cells of a plant, constitutive promoters are utilized. Additional regulatory sequences upstream and/or downstream from the core promoter sequence may be included in expression constructs of transformation vectors to bring about varying levels of expression of heterologous nucleotide sequences in a transgenic plant.

[0173] A variety of 5' and 3' transcriptional regulatory sequences are available for use in the present invention. Transcriptional terminators are responsible for the termination of transcription and correct mRNA polyadenylation. The 3' nontranslated regulatory DNA sequence preferably includes from about 50 to about 1,000, more preferably about 100 to about 1,000, nucleotide base pairs and contains plant transcriptional and translational termination sequences. Appropriate transcriptional terminators and those which are known to function in plants include the CaMV 35S terminator, the tml terminator, the nopaline synthase terminator, the pea rbcS E9 terminator, the terminator for the T7 transcript from the octopine synthase gene of *Agrobacterium tumefaciens*, and the 3' end of the protease inhibitor I or II genes

from potato or tomato, although other 3' elements known to those of skill in the art can also be employed. Alternatively, one also could use a gamma coixin, oleosin 3 or other terminator from the genus Coix.

[0174] Preferred 3' elements include those from the nopaline synthase gene of *Agrobacterium tumefaciens* (Bevan 1983), the terminator for the T7 transcript from the octopine synthase gene of *Agrobacterium tumefaciens*, and the 3' end of the protease inhibitor I or II genes from potato or tomato.

[0175] As the DNA sequence between the transcription initiation site and the start of the coding sequence, i.e., the untranslated leader sequence, can influence gene expression, one may also wish to employ a particular leader sequence. Preferred leader sequences are contemplated to include those which include sequences predicted to direct optimum expression of the attached gene, i.e., to include a preferred consensus leader sequence which may increase or maintain mRNA stability and prevent inappropriate initiation of translation. The choice of such sequences will be known to those of skill in the art in light of the present disclosure. Sequences that are derived from genes that are highly expressed in plants will be most preferred.

[0176] Preferred regulatory elements also include the 5'-untranslated region, introns and the 3'-untranslated region of genes. Such sequences that have been found to enhance gene expression in transgenic plants include intron sequences (e.g., from *Adh1*, *bronze1*, *actin1*, *actin 2* (WO 00/760067), or the sucrose synthase intron; see: *The Maize Handbook*, Chapter 116, Freeling and Walbot, Eds., Springer, New York (1994)) and viral leader sequences (e.g., from TMV, MCMV and AMV; Gallie 1987). For example, a number of non-translated leader sequences derived from viruses are known to enhance expression. Specifically, leader sequences from Tobacco Mosaic Virus (TMV), Maize Chlorotic Mottle Virus (MCMV), and Alfalfa Mosaic Virus (AMV) have been shown to be effective in enhancing expression (e.g., Gallie 1987; Skuzeski 1990). Other leaders known in the art include but are not limited to: Picornavirus leaders, for example, EMCV leader (Encephalomyocarditis 5' noncoding region) (Elroy-Stein 1989); Potyvirus leaders, for example, TEV leader (Tobacco Etch Virus); MDMV leader (Maize Dwarf Mosaic Virus); Human immunoglobulin heavy-chain binding protein (BiP) leader, (Macejak 1991); Untranslated leader from the coat protein mRNA of alfalfa mosaic virus (AMV RNA 4), (Jobling 1987; Tobacco mosaic virus leader (TMV), (Gallie 1989; and Maize Chlorotic Mottle Virus leader (MCMV) (Lommel 1991. See also, Della-Cioppa 1987. Regulatory elements such as *Adh* intron 1 (Callis 1987), sucrose synthase intron (Vasil 1989) or TMV omega element (Gallie 1989), may further be included where desired. Especially preferred are the 5'-untranslated region, introns and the 3'-untranslated region from the gene described by the GenBank *Arabidopsis thaliana* genome loci At5g58580, or of a functional equivalent thereof.

[0177] Additional preferred regulatory elements are enhancer sequences or polyadenylation sequences. Preferred polyadenylation sequences are those from plant genes or *Agrobacterium* T-DNA genes (such as for example the terminator sequences of the OCS (octopine synthase) or NOS (nopaline synthase) genes).

[0178] Examples of enhancers include elements from the CaMV 35S promoter, octopine synthase genes (Ellis et al., 1987), the rice *actin I* gene, the maize alcohol dehydrogenase gene (Callis 1987), the maize *shrunken I* gene (Vasil 1989), TMV Omega element (Gallie 1989) and promoters from non-plant eukaryotes (e.g. yeast; Ma 1988). Vectors for use in accordance with the present invention may be constructed to include the ocs enhancer element. This element was first identified as a 16 bp palindromic enhancer from the octopine synthase (*ocs*) gene of *utiliane* (Ellis 1987), and is present in at least 10 other promoters (Bouchez 1989). The use of an enhancer element, such as the *ocs* elements and particularly multiple copies of the element, will act to increase the level of transcription from adjacent promoters when applied in the context of plant transformation.

[0179] An expression cassette of the invention (or a vector derived therefrom) may comprise additional functional elements, which are to be understood in the broad sense as all elements which influence construction, propagation, or function of an expression cassette or a vector or a transgenic organism comprising them. Such functional elements may include origin of replications (to allow replication in bacteria; for the ORI of pBR322 or the P15A ori; Sambrook 1989), or elements required for *Agrobacterium* T-DNA transfer (such as for example the left and/or right border of the T-DNA).

[0180] Ultimately, the most desirable DNA segments for introduction into, for example, a dicot genome, may be homologous genes or gene families which encode a desired trait (e.g., increased yield per acre) and which are introduced under the control of novel promoters or enhancers, etc., or perhaps even homologous or tissue specific (e.g., root-, collar/sheath-, whorl-, stalk-, earshank-, kernel- or leaf-specific) promoters or control elements. Indeed, it is envisioned that a particular use of the present invention will be the expression of a gene in a guard cell-preferential or guard cell-specific manner.

[0181] Additionally, vectors may be constructed and employed in the intracellular targeting of a specific gene product within the cells of a transgenic plant or in directing a protein to the extracellular environment. This will generally be achieved by joining a DNA sequence encoding a transit or signal peptide sequence to the coding sequence of a particular gene. The resultant transit or signal peptide will transport the protein to a particular intracellular or extracellular destination, respectively, and will then be post-translationally removed. Transit or signal peptides act by facilitating the transport of proteins through intracellular membranes, e.g., vacuole, vesicle, plastid and mitochondrial membranes, whereas signal peptides direct proteins through the extracellular membrane.

[0182] A particular example of such a use concerns the direction of a herbicide resistance gene, such as the EPSPS

gene, to a particular organelle such as the chloroplast rather than to the cytoplasm. This is exemplified by the use of the *rbcS* transit peptide which confers plastid-specific targeting of proteins. In addition, it is proposed that it may be desirable to target certain genes responsible for male sterility to the mitochondria, or to target certain genes for resistance to phytopathogenic organisms to the extracellular spaces, or to target proteins to the vacuole.

5 **[0183]** By facilitating the transport of the protein into compartments inside and outside the cell, these sequences may increase the accumulation of gene product protecting them from proteolytic degradation. These sequences also allow for additional mRNA sequences from highly expressed genes to be attached to the coding sequence of the genes. Since mRNA being translated by ribosomes is more stable than naked mRNA, the presence of translatable mRNA in front of
10 the gene may increase the overall stability of the mRNA transcript from the gene and thereby increase synthesis of the gene product. Since transit and signal sequences are usually post-translationally removed from the initial translation product, the use of these sequences allows for the addition of extra translated sequences that may not appear on the final polypeptide. Targeting of certain proteins may be desirable in order to enhance the stability of the protein (US 5,545,818).

15 **[0184]** It may be useful to target DNA itself within a cell. For example, it may be useful to target introduced DNA to the nucleus as this may increase the frequency of transformation. Within the nucleus itself it would be useful to target a gene in order to achieve site specific integration. For example, it would be useful to have an gene introduced through transformation replace an existing gene in the cell. Other elements include those that can be regulated by endogenous or exogenous agents, e.g., by zinc finger proteins, including naturally occurring zinc finger proteins or chimeric zinc
20 finger proteins (see, e.g., US 5,789,538, WO 99/48909; WO 99/45132; WO 98/53060; WO 98/53057; WO 98/53058; WO 00/23464; WO 95/19431; and WO 98/54311) or myb-like transcription factors. For example, a chimeric zinc finger protein may include amino acid sequences which bind to a specific DNA sequence (the zinc finger) and amino acid sequences that activate (e.g., GAL 4 sequences) or repress the transcription of the sequences linked to the specific DNA sequence.

25 **[0185]** It is one of the objects of the present invention to provide recombinant DNA molecules comprising a nucleotide sequence according to the invention operably linked to a nucleotide segment of interest.

30 **[0186]** A nucleotide segment of interest is reflective of the commercial markets and interests of those involved in the development of the crop. Crops and markets of interest changes, and as developing nations open up world markets, new crops and technologies will also emerge. In addition, as the understanding of agronomic traits and characteristics such as yield and heterosis increase, the choice of genes for transformation will change accordingly. General categories of nucleotides of interest include, for example, genes involved in information, such as zinc fingers, those involved in
35 communication, such as kinases, and those involved in housekeeping, such as heat shock proteins. More specific categories of transgenes, for example, include genes encoding important traits for agronomics, insect resistance, disease resistance, herbicide resistance, sterility, grain characteristics, and commercial products. Genes of interest include, generally, those involved in starch, oil, carbohydrate, or nutrient metabolism, as well as those affecting kernel size, sucrose loading, zinc finger proteins, see, e.g., US 5,789,538, WO 99/48909; WO 99/45132; WO 98/53060; WO 98/53057;
40 WO 98/53058; WO 00/23464; WO 95/19431; and WO 98/54311, and the like.

[0187] One skilled in the art recognizes that the expression level and regulation of a transgene in a plant can vary significantly from line to line. Thus, one has to test several lines to find one with the desired expression level and regulation. Once a line is identified with the desired regulation specificity of a chimeric Cre transgene, it can be crossed
45 with lines carrying different inactive replicons or inactive transgene for activation.

[0188] Other sequences which may be linked to the gene of interest which encodes a polypeptide are those which can target to a specific organelle, e.g., to the mitochondria, nucleus, or plastid, within the plant cell. Targeting can be achieved by providing the polypeptide with an appropriate targeting peptide sequence, such as a secretory signal peptide (for secretion or cell wall or membrane targeting, a plastid transit peptide, a chloroplast transit peptide, e.g., the chlorophyll
50 a/b binding protein, a mitochondrial target peptide, a vacuole targeting peptide, or a nuclear targeting peptide, and the like. For example, the small subunit of ribulose biphosphate carboxylase transit peptide, the EPSPS transit peptide or the dihydrodipicolinic acid synthase transit peptide may be used. For examples of plastid organelle targeting sequences (see WO 00/12732). Plastids are a class of plant organelles derived from proplastids and include chloroplasts, leucoplasts, amyloplasts, and chromoplasts. The plastids are major sites of biosynthesis in plants. In addition to photosynthesis in
55 the chloroplast, plastids are also sites of lipid biosynthesis, nitrate reduction to ammonium, and starch storage. And while plastids contain their own circular, genome, most of the proteins localized to the plastids are encoded by the nuclear genome and are imported into the organelle from the cytoplasm.

[0189] Transgenes used with the present invention will often be genes that direct the expression of a particular protein or polypeptide product, but they may also be non-expressible DNA segments, e.g., transposons such as Ds that do not
60 direct their own transposition. As used herein, an "expressible gene" is any gene that is capable of being transcribed into RNA (e.g., mRNA, antisense RNA, etc.) or translated into a protein, expressed as a trait of interest, or the like, etc., and is not limited to selectable, screenable or non-selectable marker genes. The invention also contemplates that, where both an expressible gene that is not necessarily a marker gene is employed in combination with a marker gene, one

may employ the separate genes on either the same or different DNA segments for transformation. In the latter case, the different vectors are delivered concurrently to recipient cells to maximize cotransformation.

[0190] The choice of the particular DNA segments to be delivered to the recipient cells will often depend on the purpose of the transformation. One of the major purposes of transformation of crop plants is to add some commercially desirable, agronomically important traits to the plant. Such traits include, but are not limited to, herbicide resistance or tolerance; insect resistance or tolerance; disease resistance or tolerance (viral, bacterial, fungal, nematode); stress tolerance and/or resistance, as exemplified by resistance or tolerance to drought, heat, chilling, freezing, excessive moisture, salt stress; oxidative stress; increased yields; food content and makeup; physical appearance; male sterility; drydown; standability; prolificacy; starch properties; oil quantity and quality; and the like. One may desire to incorporate one or more genes conferring any such desirable trait or traits, such as, for example, a gene or genes encoding pathogen resistance.

[0191] In certain embodiments, the present invention contemplates the transformation of a recipient cell with more than one advantageous transgene. Two or more transgenes can be supplied in a single transformation event using either distinct transgene-encoding vectors, or using a single vector incorporating two or more gene coding sequences. For example, plasmids bearing the bar and aroA expression units in either convergent, divergent, or colinear orientation, are considered to be particularly useful. Further preferred combinations are those of an insect resistance gene, such as a Bt gene, along with a protease inhibitor gene such as pinII, or the use of bar in combination with either of the above genes. Of course, any two or more transgenes of any description, such as those conferring herbicide, insect, disease (viral, bacterial, fungal, nematode) or drought resistance, male sterility, drydown, standability, prolificacy, starch properties, oil quantity and quality, or those increasing yield or nutritional quality may be employed as desired.

1. Exemplary Transgenes

[0192] The following examples are especially useful for expression in guard-cells, but may be also expressed by additional expression cassettes comprised in an expression construct or vector of the invention.

1.1 Insect and Disease Resistance

[0193] An important aspect of the present invention concerns the introduction of insect or disease resistance-conferring genes into plants. Certain pathogens, such as fungi, may enter the plant through the stomata. Modification of the guard cells may be useful to prohibit this entrance. Many other pathogen-resistance genes suitable for transgenic expression (such as Bt genes, Watrud 1985) are known in the art.

1.2 Environment or Stress Resistance

[0194] Stress-tolerance is an especially preferred trait which can be achieved by guard-cell-specific gene expression (see above for examples). Improvement of a plant's ability to tolerate various environmental stresses such as, but not limited to, drought, excess moisture, chilling, freezing, high temperature, salt, and oxidative stress, can also be effected through expression of heterologous, or overexpression of homologous genes. Benefits may be realized in terms of increased resistance to freezing temperatures through the introduction of an "antifreeze" protein such as that of the Winter Flounder (Cutler 1989) or synthetic gene derivatives thereof. Improved chilling tolerance may also be conferred through increased expression of glycerol-3-phosphate acetyltransferase in chloroplasts (Murata 1992; Wolter 1992). Resistance to oxidative stress (often exacerbated by conditions such as chilling temperatures in combination with high light intensities) can be conferred by expression of superoxide dismutase (Gupta 1993), and may be improved by glutathione reductase (Bowler 1992). Such strategies may allow for tolerance to freezing in newly emerged fields as well as extending later maturity higher yielding varieties to earlier relative maturity zones.

[0195] Expression of novel genes that favorably effect plant water content, total water potential, osmotic potential, and turgor can enhance the ability of the plant to tolerate drought. As used herein, the terms "drought resistance" and "drought tolerance" are used to refer to a plants increased resistance or tolerance to stress induced by a reduction in water availability, as compared to normal circumstances, and the ability of the plant to function and survive in lower-water environments, and perform in a relatively superior manner. In this aspect of the invention it is proposed, for example, that the expression of a gene encoding the biosynthesis of osmotically-active solutes can impart protection against drought. Within this class of genes are DNAs encoding mannitol dehydrogenase (Lee and Saier, 1982) and trehalose-6-phosphate synthase (Kaasen 1992). Through the subsequent action of native phosphatases in the cell or by the introduction and coexpression of a specific phosphatase, these introduced genes will result in the accumulation of either mannitol or trehalose, respectively, both of which have been well documented as protective compounds able to mitigate the effects of stress. Mannitol accumulation in transgenic tobacco has been verified and preliminary results indicate that plants expressing high levels of this metabolite are able to tolerate an applied osmotic stress (Tarczynski 1992).

[0196] Similarly, the efficacy of other metabolites in protecting either enzyme function (e.g. alanopine or propionic

acid) or membrane integrity (e.g., alanopine) has been documented (Loomis 1989), and therefore expression of gene encoding the biosynthesis of these compounds can confer drought resistance in a manner similar to or complimentary to mannitol. Other examples of naturally occurring metabolites that are osmotically active and/or provide some direct protective effect during drought and/or desiccation include sugars and sugar derivatives such as fructose, erythritol (Coxson 1992), sorbitol, dulcitol (Karsten 1992), glucosylglycerol (Reed 1984; Erdmann 1992), sucrose, stachyose (Koster & Leopold 1988; Blackman 1992), ononitol and pinitol (Vernon & Bohnert 1992), and raffinose (Bemal-Lugo & Leopold 1992). Other osmotically active solutes which are not sugars include, but are not limited to, proline and glycine-betaine (Wyn-Jones and Storey, 1981). Continued canopy growth and increased reproductive fitness during times of stress can be augmented by introduction and expression of genes such as those controlling the osmotically active compounds discussed above and other such compounds, as represented in one exemplary embodiment by the enzyme myoinositol 0-methyltransferase.

[0197] It is contemplated that the expression of specific proteins may also increase drought tolerance. Three classes of Late Embryogenic Proteins have been assigned based on structural similarities (see Dure 1989). All three classes of these proteins have been demonstrated in maturing (i.e., desiccating) seeds. Within these 3 types of proteins, the Type-II (dehydrin-type) have generally been implicated in drought and/or desiccation tolerance in vegetative plant parts (e.g., Mundy and Chua, 1988; Piatkowski 1990; Yamaguchi-Shinozaki 1992). Recently, expression of a Type-III LEA (HVA-1) in tobacco was found to influence plant height, maturity and drought tolerance (Fitzpatrick, 1993). Expression of structural genes from all three groups may therefore confer drought tolerance. Other types of proteins induced during water stress include thiol proteases, aldolases and transmembrane transporters (Guerrero 1990), which may confer various protective and/or repair-type functions during drought stress. The expression of a gene that effects lipid biosynthesis and hence membrane composition can also be useful in conferring drought resistance on the plant.

[0198] Many genes that improve drought resistance have complementary modes of action. Thus, combinations of these genes might have additive and/or synergistic effects in improving drought resistance in maize. Many of these genes also improve freezing tolerance (or resistance); the physical stresses incurred during freezing and drought are similar in nature and may be mitigated in similar fashion. Benefit may be conferred via constitutive or tissue-specific expression of these genes, but the preferred means of expressing these novel genes may be through the use of a turgor-induced promoter (such as the promoters for the turgor-induced genes described in Guerrero et al. 1990 and Shagan 1993). Spatial and temporal expression patterns of these genes may enable maize to better withstand stress.

[0199] Expression of genes that are involved with specific morphological traits that allow for increased water extractions from drying soil would be of benefit. For example, introduction and expression of genes that alter root characteristics may enhance water uptake. Expression of genes that enhance reproductive fitness during times of stress would be of significant value. For example, expression of DNAs that improve the synchrony of pollen shed and receptiveness of the female flower parts, i.e., silks, would be of benefit. In addition, expression of genes that minimize kernel abortion during times of stress would increase the amount of grain to be harvested and hence be of value. Regulation of cytokinin levels in monocots, such as maize, by introduction and expression of an isopentenyl transferase gene with appropriate regulatory sequences can improve monocot stress resistance and yield (Gan 1995).

[0200] Given the overall role of water in determining yield, it is contemplated that enabling plants to utilize water more efficiently, through the introduction and expression of novel genes, will improve overall performance even when soil water availability is not limiting. By introducing genes that improve the ability of plants to maximize water usage across a full range of stresses relating to water availability, yield stability or consistency of yield performance may be realized.

[0201] Improved protection of the plant to abiotic stress factors such as drought, heat or chill, can also be achieved - for example - by overexpressing antifreeze polypeptides from *Myoxocephalus Scorpius* (WO 00/00512), *Myoxocephalus octodecemspinosus*, the *Arabidopsis thaliana* transcription activator CBF1, glutamate dehydrogenases (WO 97/12983, WO 98/11240), calcium-dependent protein kinase genes (WO 98/26045), calcineurins (WO 99/05902), casein kinase from yeast (WO 02/052012), farnesyltransferases (WO 99/06580; Pei ZM et al. (1998) *Science* 282:287-290), ferritin (Deak M et al. (1999) *Nature Biotechnology* 17:192-196), oxalate oxidase (WO 99/04013; Dunwell JM (1998) *Biotechnol Genet Eng Rev* 15:1-32), DREB1A factor ("dehydration response element B 1A"; Kasuga M et al. (1999) *Nature Biotech* 17:276-286), genes of mannitol or trehalose synthesis such as trehalose-phosphate synthase or trehalose-phosphate phosphatase (WO 97/42326) or by inhibiting genes such as trehalase (WO 97/50561).

1.3 Plant Agronomic Characteristics

[0202] As described above various agronomic characteristics can be improved by guard cell-specific gene expression. For example one can achieve increased yield by modified gas exchange and transpiration characteristics which improve carbon dioxide supply and eventually enhance the efficiency of photosynthesis. Furthermore stress resistance (especially against draught) can be enhanced. Two of the factors determining where plants can be grown are the average daily temperature during the growing season and the length of time between frosts. Within the areas where it is possible to grow a particular plant, there are varying limitations on the maximal time it is allowed to grow to maturity and be harvested.

The plant to be grown in a particular area is selected for its ability to mature and dry down to harvestable moisture content within the required period of time with maximum possible yield. Therefore, plant of varying maturities are developed for different growing locations. Apart from the need to dry down sufficiently to permit harvest is the desirability of having maximal drying take place in the field to minimize the amount of energy required for additional drying post-harvest. Also the more readily the grain can dry down, the more time there is available for growth and kernel fill. Genes that influence maturity and/or dry down can be identified and introduced into plant lines using transformation techniques to create new varieties adapted to different growing locations or the same growing location but having improved yield to moisture ratio at harvest. Expression of genes that are involved in regulation of plant development may be especially useful, e.g., the liguleless and rough sheath genes that have been identified in plants.

[0203] Genes may be introduced into plants that would improve standability and other plant growth characteristics. For example, expression of novel genes which confer stronger stalks, improved root systems, or prevent or reduce ear droppage would be of great value to the corn farmer. Introduction and expression of genes that increase the total amount of photoassimilate available by, for example, increasing light distribution and/or interception would be advantageous. In addition the expression of genes that increase the efficiency of photosynthesis and/or the leaf canopy would further increase gains in productivity. Such approaches would allow for increased plant populations in the field.

[0204] Delay of late season vegetative senescence would increase the flow of assimilate into the grain and thus increase yield. Overexpression of genes within plants that are associated with "stay green" or the expression of any gene that delays senescence would be advantageous. For example, a non-yellowing mutant has been identified in *Festuca pratensis* (Davies 1990). Expression of this gene as well as others may prevent premature breakdown of chlorophyll and thus maintain canopy function.

1.4. Non-Protein-Expressing Sequences

1.4.1 RNA-Expressing

[0205] DNA may be introduced into plants for the purpose of expressing RNA transcripts that function to affect plant phenotype yet are not translated into protein. Two examples are antisense RNA and RNA with ribozyme activity. Both may serve possible functions in reducing or eliminating expression of native or introduced plant genes.

[0206] Genes may be constructed or isolated, which when transcribed, produce antisense RNA or double-stranded RNA that is complementary to all or part(s) of a targeted messenger RNA(s). The antisense RNA reduces production of the polypeptide product of the messenger RNA. The polypeptide product may be any protein encoded by the plant genome. The aforementioned genes will be referred to as antisense genes. An antisense gene may thus be introduced into a plant by transformation methods to produce a novel transgenic plant with reduced expression of a selected protein of interest. For example, the protein may be an enzyme that catalyzes a reaction in the plant. Reduction of the enzyme activity may reduce or eliminate products of the reaction which include any enzymatically synthesized compound in the plant such as fatty acids, amino acids, carbohydrates, nucleic acids and the like. Alternatively, the protein may be a storage protein, such as a zein, or a structural protein, the decreased expression of which may lead to changes in seed amino acid composition or plant morphological changes respectively. The possibilities cited above are provided only by way of example and do not represent the full range of applications.

[0207] Expression of antisense-RNA or double-stranded RNA by one of the expression cassettes of the invention is especially preferred. Also expression of sense RNA can be employed for gene silencing (co-suppression). This RNA is preferably a non-translatable RNA. Gene regulation by double-stranded RNA ("double-stranded RNA interference"; dsRNAi) is well known in the art and described for various organism including plants (e.g., Matzke 2000; Fire A et al 1998; WO 99/32619; WO 99/53050; WO 00/68374; WO 00/44914; WO 00/44895; WO 00/49035; WO 00/63364).

[0208] Genes may also be constructed or isolated, which when transcribed produce RNA enzymes, or ribozymes, which can act as endoribonucleases and catalyze the cleavage of RNA molecules with selected sequences. The cleavage of selected messenger RNA's can result in the reduced production of their encoded polypeptide products. These genes may be used to prepare novel transgenic plants which possess them. The transgenic plants may possess reduced levels of polypeptides including but not limited to the polypeptides cited above that may be affected by antisense RNA.

[0209] It is also possible that genes may be introduced to produce novel transgenic plants which have reduced expression of a native gene product by a mechanism of cosuppression. It has been demonstrated in tobacco, tomato, and petunia (Goring 1991; Smith 1990; Napoli 1990; van der Krol 1990) that expression of the sense transcript of a native gene will reduce or eliminate expression of the native gene in a manner similar to that observed for antisense genes. The introduced gene may encode all or part of the targeted native protein but its translation may not be required for reduction of levels of that native protein.

1.4.2 Non-RNA-Expressing

[0210] For example, DNA elements including those of transposable elements such as Ds, Ac, or Mu, may be, inserted into a gene and cause mutations. These DNA elements may be inserted in order to inactivate (or activate) a gene and thereby "tag" a particular trait. In this instance the transposable element does not cause instability of the tagged mutation, because the utility of the element does not depend on its ability to move in the genome. Once a desired trait is tagged, the introduced DNA sequence may be used to clone the corresponding gene, e.g., using the introduced DNA sequence as a PCR primer together with PCR gene cloning techniques (Shapiro, 1983; Dellaporta 1988). Once identified, the entire gene(s) for the particular trait, including control or regulatory regions where desired may be isolated, cloned and manipulated as desired. The utility of DNA elements introduced into an organism for purposes of gene tagging is independent of the DNA sequence and does not depend on any biological activity of the DNA sequence, i.e., transcription into RNA or translation into protein. The sole function of the DNA element is to disrupt the DNA sequence of a gene.

[0211] It is contemplated that unexpressed DNA sequences, including novel synthetic sequences could be introduced into cells as proprietary "labels" of those cells and plants and seeds thereof. It would not be necessary for a label DNA element to disrupt the function of a gene endogenous to the host organism, as the sole function of this DNA would be to identify the origin of the organism. For example, one could introduce a unique DNA sequence into a plant and this DNA element would identify all cells, plants, and progeny of these cells as having arisen from that labeled source. It is proposed that inclusion of label DNAs would enable one to distinguish proprietary germplasm or germplasm derived from such, from unlabelled germplasm.

[0212] Another possible element which may be introduced is a matrix attachment region element (MAR), such as the chicken lysozyme A element (Stief 1989), which can be positioned around an expressible gene of interest to effect an increase in overall expression of the gene and diminish position dependant effects upon incorporation into the plant genome (Stief 1989; Phi-Van 1990).

[0213] Further nucleotide sequences of interest that may be contemplated for use within the scope of the present invention in operable linkage with the promoter sequences according to the invention are isolated nucleic acid molecules, e.g., DNA or RNA, comprising a plant nucleotide sequence according to the invention comprising an open reading frame that is preferentially expressed in a specific tissue, i.e., seed-, root, green tissue (leaf and stem), panicle-, or pollen, or is expressed constitutively.

2. Marker Genes

[0214] The following examples are especially useful in combination with a constitutive promoter for incorporation in an expression construct or vector of the invention. However also a guard cell-specific or preferential expression may be useful.

[0215] In order to improve the ability to identify transformants, one may desire to employ a selectable or screenable marker gene as, or in addition to, the expressible gene of interest. "Marker genes" are genes that impart a distinct phenotype to cells expressing the marker gene and thus allow such transformed cells to be distinguished from cells that do not have the marker. Such genes may encode either a selectable or screenable marker, depending on whether the marker confers a trait which one can 'select' for by chemical means, i.e., through the use of a selective agent (e.g., a herbicide, antibiotic, or the like), or whether it is simply a trait that one can identify through observation or testing, i.e., by 'screening' (e.g., the R-locus trait, the green fluorescent protein (GFP)). Of course, many examples of suitable marker genes are known to the art and can be employed in the practice of the invention.

[0216] Included within the terms selectable or screenable marker genes are also genes which encode a "secretable marker" whose secretion can be detected as a means of identifying or selecting for transformed cells. Examples include markers which encode a secretable antigen that can be identified by antibody interaction, or even secretable enzymes which can be detected by their catalytic activity. Secretable proteins fall into a number of classes, including small, diffusible proteins detectable, e.g., by ELISA; small active enzymes detectable in extracellular solution (e.g., alpha-amylase, beta-lactamase, phosphinothricin acetyltransferase); and proteins that are inserted or trapped in the cell wall (e.g., proteins that include a leader sequence such as that found in the expression unit of extensin or tobacco PR-S).

[0217] With regard to selectable secretable markers, the use of a gene that encodes a protein that becomes sequestered in the cell wall, and which protein includes a unique epitope is considered to be particularly advantageous. Such a secreted antigen marker would ideally employ an epitope sequence that would provide low background in plant tissue, a promoter-leader sequence that would impart efficient expression and targeting across the plasma membrane, and would produce protein that is bound in the cell wall and yet, accessible to antibodies. A normally secreted wall protein modified to include a unique epitope would satisfy all such requirements.

[0218] One example of a protein suitable for modification in this manner is extensin, or hydroxyproline rich glycoprotein (HPRG). For example, the maize HPRG (Steifel 1990) molecule is well characterized in terms of molecular biology, expression and protein structure. However, any one of a variety of utilane and/or glycine-rich wall proteins (Keller 1989)

could be modified by the addition of an antigenic site to create a screenable marker.

[0219] One exemplary embodiment of a secretable screenable marker concerns the use of a maize sequence encoding the wall protein HPRG, modified to include a 15 residue epitope from the pro-region of murine interleukin, however, virtually any detectable epitope may be employed in such embodiments, as selected from the extremely wide variety of antigen-antibody combinations known to those of skill in the art. The unique extracellular epitope can then be straightforwardly detected using antibody labeling in conjunction with chromogenic or fluorescent adjuncts.

[0220] Elements of the present disclosure may be exemplified in detail through the use of the bar and/or GUS genes, and also through the use of various other markers. Of course, in light of this disclosure, numerous other possible selectable and/or screenable marker genes will be apparent to those of skill in the art in addition to the one set forth herein below. Therefore, it will be understood that the following discussion is exemplary rather than exhaustive. In light of the techniques disclosed herein and the general recombinant techniques which are known in the art, the present invention renders possible the introduction of any gene, including marker genes, into a recipient cell to generate a transformed plant.

2.1 Selectable Markers

[0221] Various selectable markers are known in the art suitable for plant transformation. Such markers may include but are not limited to:

2.1.1 Negative selection markers

[0222] Negative selection markers confer a resistance to a biocidal compound such as a metabolic inhibitor (e.g., 2-deoxyglucose-6-phosphate, WO 98/45456), antibiotics (e.g., kanamycin, G 418, bleomycin or hygromycin) or herbicides (e.g., phosphinothricin or glyphosate). Transformed plant material (e.g., cells, tissues or plantlets), which express marker genes, are capable of developing in the presence of concentrations of a corresponding selection compound (e.g., antibiotic or herbicide) which suppresses growth of an untransformed wild type tissue. Especially preferred negative selection markers are those which confer resistance to herbicides. These markers can also be employed as traits for herbicide resistance. Examples which may be mentioned are:

- Phosphinothricin acetyltransferases (PAT; also named Bialophos[®] resistance; bar; de Block 1987; Vasil 1992, 1993; Weeks 1993; Becker 1994; Nehra 1994; Wan & Lemaux 1994; EP 0 333 033; US 4,975,374). Preferred are the bar gene from *Streptomyces hygrosopicus* or the pat gene from *Streptomyces viridochromogenes*. PAT inactivates the active ingredient in the herbicide Bialophos, phosphinothricin (PPT). PPT inhibits glutamine synthetase, (Murakami 1986; Twell 1989) causing rapid accumulation of ammonia and cell death..
- altered 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) conferring resistance to Glyphosate[®] (N-(phosphonomethyl)glycine) (Hinchee 1988; Shah 1986; Della-Cioppa 1987). Where a mutant EPSP synthase gene is employed, additional benefit may be realized through the incorporation of a suitable chloroplast transit peptide, CTP (EP-A1 0 218 571).
- Glyphosate[®] degrading enzymes (Glyphosate[®] oxidoreductase; gox),
- Dalapon[®] inactivating dehalogenases (deh)
- sulfonyleurea- and/or imidazolinone-inactivating acetolactate synthases (*ahas* or ALS; for example mutated *ahas*/ALS variants with, for example, the S4, X112, XA17, and/or Hra mutation (EP-A1 154 204)
- Bromoxynil[®] degrading nitrilases (bxn; Stalker 1988)
- Kanamycin- or. geneticin (G418) resistance genes (NPTII; NPT or neo; Potrykus 1985) coding e.g., for neomycin phosphotransferases (Fraley 1983; Nehra 1994)
- 2-Desoxyglucose-6-phosphate phosphatase (DOG^R1-Gene product; WO 98/45456; EP 0 807 836) conferring resistance against 2-desoxyglucose (Randez-Gil 1995).
- hygromycin phosphotransferase (HPT), which mediates resistance to hygromycin (Vanden Elzen 1985).
- altered dihydrofolate reductase (Eichholtz 1987) conferring resistance against methotrexat (Thillet 1988);
- mutated anthranilate synthase genes that confers resistance to 5-methyl tryptophan.

[0223] Additional negative selectable marker genes of bacterial origin that confer resistance to antibiotics include the *aadA* gene, which confers resistance to the antibiotic spectinomycin, gentamycin acetyl transferase, streptomycin phosphotransferase (SPT), aminoglycoside-3-adenyl transferase and the bleomycin resistance determinant (Hayford 1988; Jones 1987; Svab 1990; Hille 1986).

[0224] Especially preferred are negative selection markers that confer resistance against the toxic effects imposed by D-amino acids like e.g., D-alanine and D-serine (WO 03/060133; Erikson 2004). Especially preferred as negative selection marker in this contest are the *daol* gene (EC: 1.4. 3.3 : GenBank Acc.-No.: U60066) from the yeast *Rhodotorula gracilis* (*Rhodospordium toruloides*) and the *E. coli* gene *dsdA* (D-serine dehydratase (D-serine deaminase) [EC: 4.3.

1.18; GenBank Acc.-No.: J01603).

[0225] Transformed plant material (e.g., cells, embryos, tissues or plantlets) which express such marker genes are capable of developing in the presence of concentrations of a corresponding selection compound (e.g., antibiotic or herbicide) which suppresses growth of an untransformed wild type tissue. The resulting plants can be bred and hybridized in the customary fashion. Two or more generations should be grown in order to ensure that the genomic integration is stable and hereditary. Corresponding methods are described (Jenes 1993; Potrykus 1991).

[0226] Furthermore, reporter genes can be employed to allow visual screening, which may or , may not (depending on the type of reporter gene) require supplementation with a substrate as a selection compound.

[0227] Various time schemes can be employed for the various negative selection marker genes. In case of resistance genes (e.g., against herbicides or D-amino acids) selection is preferably applied throughout callus induction phase for about 4 weeks and beyond at least 4 weeks into regeneration. Such a selection scheme can be applied for all selection regimes. It is furthermore possible (although not explicitly preferred) to remain the selection also throughout the entire regeneration scheme including rooting.

[0228] For example, with the phosphinotricin resistance gene (*bar*) as the selective marker, phosphinotricin at a concentration of from about 1 to 50 mg/l may be included in the medium. For example, with the *dao1* gene as the selective marker, D-serine or D-alanine at a concentration of from about 3 to 100 mg/l may be included in the medium. Typical concentrations for selection are 20 to 40 mg/l. For example, with the mutated *ahas* genes as the selective marker, PURSUIT™ at a concentration of from about 3 to 100 mg/l may be included in the medium. Typical concentrations for selection are 20 to 40 mg/l.

2.1.2 Positive selection marker

[0229] Furthermore, positive selection marker can be employed. Genes like isopentenyltransferase from *Agrobacterium tumefaciens* (strain:PO22; GenBank Acc.-No.: AB025109) may - as a key enzyme of the cytokinin biosynthesis - facilitate regeneration of transformed plants (e.g., by selection on cytokinin-free medium). Corresponding selection methods are described (Ebinuma 2000a,b). Additional positive selection markers, which confer a growth advantage to a transformed plant in comparison with a non-transformed one, are described e.g., in EP-A 0 601 092. Growth stimulation selection markers may include (but shall not be limited to) β -Glucuronidase (in combination with e.g., a cytokinin glucuronide), mannose-6-phosphate isomerase (in combination with mannose), UDP-galactose-4-epimerase (in combination with e.g., galactose), wherein mannose-6-phosphate isomerase in combination with mannose is especially preferred.

2.1.3 Counter-selection marker

[0230] Counter-selection markers are especially suitable to select organisms with defined deleted sequences comprising said marker (Koprek 1999). Examples for counter- selection marker comprise thymidin kinases (TK), cytosine deaminases (Gleave 1999; Perera 1993; Stougaard 1993), cytochrom P450 proteins (Koprek 1999), haloalkan dehalogenases (Naested 1999), *iaaH* gene products (Sundaresan 1995), cytosine deaminase *codA* (Schlaman & Hooykaas 1997), *tms2* gene products (Fedoroff & Smith 1993), or *a-naphthalene acetamide* (NAM; Depicker 1988). Counter selection markers may be useful in the construction of transposon tagging lines. For example, by marking an autonomous transposable element such as *Ac*, *Master Mu*, or *En/Spn* with a counter selection marker, one could select for transformants in which the autonomous element is not stably integrated into the genome. This would be desirable, for example, when transient expression of the autonomous element is desired to activate in trans the transposition of a defective transposable element, such as *Ds*, but stable integration of the autonomous element is not desired. The presence of the autonomous element may not be desired in order to stabilize the defective element, i.e., prevent it from further transposing. However, it is proposed that if stable integration of an autonomous transposable element is desired in a plant the presence of a negative selectable marker may make it possible to eliminate the autonomous element during the breeding process.

2.2. Screenable Markers

[0231] Screenable markers that may be employed include, but are not limited to, a beta-glucuronidase (GUS) or *uidA* gene which encodes an enzyme for which various chromogenic substrates are known; an R-locus gene, which encodes a product that regulates the production of anthocyanin pigments (red color) in plant tissues (Dellaporta 1988); a beta-lactamase gene (Sutcliffe 1978), which encodes an enzyme for which various chromogenic substrates are known (e.g., PADAC, a chromogenic cephalosporin); a *xylE* gene (Zukowsky 1983) which encodes a catechol dioxygenase that can convert chromogenic catechols; an *a-amylase* gene (Ikuta 1990); a tyrosinase gene (Katz 1983) which encodes an enzyme capable of oxidizing tyrosine to DOPA and dopaquinone which in turn condenses to form the easily detectable compound melanin; β -galactosidase gene, which encodes an enzyme for which there are chromogenic substrates; a

luciferase (lux) gene (Ow 1986), which allows for bioluminescence detection; or even an aequorin gene (Prasher 1985), which may be employed in calcium-sensitive bioluminescence detection, or a green fluorescent protein gene (Niedz 1995).

[0232] Genes from the maize R gene complex are contemplated to be particularly useful as screenable markers. The R gene complex in maize encodes a protein that acts to regulate the production of anthocyanin pigments in most seed and plant tissue. A gene from the R gene complex was applied to maize transformation, because the expression of this gene in transformed cells does not harm the cells. Thus, an R gene introduced into such cells will cause the expression of a red pigment and, if stably incorporated, can be visually scored as a red sector. If a maize line carries dominant genes encoding the enzymatic intermediates in the anthocyanin biosynthetic pathway (C2, A1, A2, Bz1 and Bz2), but carries a recessive allele at the R locus, transformation of any cell from that line with R will result in red pigment formation. Exemplary lines include Wisconsin 22 which contains the rg-Stadler allele and TR112, a K55 derivative which is r-g, b, P1. Alternatively any genotype of maize can be utilized if the C1 and R alleles are introduced together.

[0233] It is further proposed that R gene regulatory regions may be employed in chimeric constructs in order to provide mechanisms for controlling the expression of chimeric genes. More diversity of phenotypic expression is known at the R locus than at any other locus (Coe 1988). It is contemplated that regulatory regions obtained from regions 5' to the structural R gene would be valuable in directing the expression of genes, e.g., insect resistance, drought resistance, herbicide tolerance or other protein coding regions. For the purposes of the present invention, it is believed that any of the various R gene family members may be successfully employed (e.g., P, S, Lc, etc.). However, the most preferred will generally be Sn (particularly Sn:bol3). Sn is a dominant member of the R gene complex and is functionally similar to the R and B loci in that Sn controls the tissue specific deposition of anthocyanin pigments in certain seedling and plant cells, therefore, its phenotype is similar to R.

[0234] A further screenable marker contemplated for use in the present invention is firefly luciferase, encoded by the lux gene. The presence of the lux gene in transformed cells may be detected using, for example, X-ray film, scintillation counting, fluorescent spectrophotometry, low-light video cameras, photon counting cameras or multiwell luminometry. It is also envisioned that this system may be developed for populational screening for bioluminescence, such as on tissue culture plates, or even for whole plant screening. Where use of a screenable marker gene such as lux or GFP is desired, benefit may be realized by creating a gene fusion between the screenable marker gene and a selectable marker gene, for example, a GFP-NPTII gene fusion. This could allow, for example, selection of transformed cells followed by screening of transgenic plants or seeds.

3. Exemplary DNA Molecules

[0235] The invention provides an isolated nucleic acid molecule, e.g., DNA or RNA, comprising a plant nucleotide sequence comprising an open reading frame that is preferentially expressed in a specific plant tissue, i.e., in seeds, roots, green tissue (leaf and stem), panicles or pollen, or is expressed constitutively, or a promoter thereof.

[0236] These promoters include, but are not limited to, constitutive, inducible, temporally regulated, developmentally regulated, spatially regulated, chemically regulated, stress-responsive, tissue-specific, viral and synthetic promoters. Promoter sequences are known to be strong or weak. A strong promoter provides for a high level of gene expression, whereas a weak promoter provides for a very low level of gene expression. An inducible promoter is a promoter that provides for the turning on and off of gene expression in response to an exogenously added agent, or to an environmental or developmental stimulus. A bacterial promoter such as the P_{tac} promoter can be induced to varying levels of gene expression depending on the level of isothiopyrogalactoside added to the transformed bacterial cells. An isolated promoter sequence that is a strong promoter for heterologous nucleic acid is advantageous because it provides for a sufficient level of gene expression to allow for easy detection and selection of transformed cells and provides for a high level of gene expression when desired.

[0237] Within a plant promoter region there are several domains that are necessary for full function of the promoter. The first of these domains lies immediately upstream of the structural gene and forms the "core promoter region" containing consensus sequences, normally 70 base pairs immediately upstream of the gene. The core promoter region contains the characteristic CAAT and TATA boxes plus surrounding sequences, and represents a transcription initiation sequence that defines the transcription start point for the structural gene.

[0238] The presence of the core promoter region defines a sequence as being a promoter: if the region is absent, the promoter is non-functional. Furthermore, the core promoter region is insufficient to provide full promoter activity. A series of regulatory sequences upstream of the core constitute the remainder of the promoter. The regulatory sequences determine expression level, the spatial and temporal pattern of expression and, for an important subset of promoters, expression under inductive conditions (regulation by external factors such as light, temperature, chemicals, hormones).

[0239] Regulated expression of the chimeric transacting viral replication protein can be further regulated by other genetic strategies. For example, Cre-mediated gene activation as described by Odell et al. 1990. Thus, a DNA fragment containing 3' regulatory sequence bound by lox sites between the promoter and the replication protein coding sequence

that blocks the expression of a chimeric replication gene from the promoter can be removed by Cre-mediated excision and result in the expression of the transacting replication gene. In this case, the chimeric Cre gene, the chimeric transacting replication gene, or both can be under the control of tissue- and developmental-specific or inducible promoters. An alternate genetic strategy is the use of tRNA suppressor gene. For example, the regulated expression of a tRNA suppressor gene can conditionally control expression of a trans-acting replication protein coding sequence containing an appropriate termination codon as described by Ulmasov et al. 1997. Again, either the chimeric tRNA suppressor gene, the chimeric transacting replication gene, or both can be under the control of tissue- and developmental-specific or inducible promoters.

[0240] Frequently it is desirable to have continuous or inducible expression of a DNA sequence throughout the cells of an organism in a tissue-independent manner. For example, increased resistance of a plant to infection by soil- and airborne-pathogens might be accomplished by genetic manipulation of the plant's genome to comprise a continuous promoter operably linked to a heterologous pathogen-resistance gene such that pathogen-resistance proteins are continuously expressed throughout the plant's tissues.

[0241] Alternatively, it might be desirable to inhibit expression of a native DNA sequence within the seeds of a plant to achieve a desired phenotype. In this case, such inhibition might be accomplished with transformation of the plant to comprise a promoter operably linked to an antisense nucleotide sequence, such that guard cell-preferential or guard cell-specific expression of the antisense sequence produces an RNA transcript that interferes with translation of the mRNA of the native DNA sequence.

[0242] To define a minimal promoter region, a DNA segment representing the promoter region is removed from the 5' region of the gene of interest and operably linked to the coding sequence of a marker (reporter) gene by recombinant DNA techniques well known to the art. The reporter gene is operably linked downstream of the promoter, so that transcripts initiating at the promoter proceed through the reporter gene. Reporter genes generally encode proteins which are easily measured, including, but not limited to, chloramphenicol acetyl transferase (CAT), beta-glucuronidase (GUS), green fluorescent protein (GFP), beta-galactosidase (beta-GAL), and luciferase.

[0243] The construct containing the reporter gene under the control of the promoter is then introduced into an appropriate cell type by transfection techniques well known to the art. To assay for the reporter protein, cell lysates are prepared and appropriate assays, which are well known in the art, for the reporter protein are performed. For example, if CAT were the reporter gene of choice, the lysates from cells transfected with constructs containing CAT under the control of a promoter under study are mixed with isotopically labeled chloramphenicol and acetyl-coenzyme A (acetyl-CoA). The CAT enzyme transfers the acetyl group from acetyl-CoA to the 2- or 3-position of chloramphenicol. The reaction is monitored by thin-layer chromatography, which separates acetylated chloramphenicol from unreacted material. The reaction products are then visualized by autoradiography.

[0244] The level of enzyme activity corresponds to the amount of enzyme that was made, which in turn reveals the level of expression from the promoter of interest. This level of expression can be compared to other promoters to determine the relative strength of the promoter under study. In order to be sure that the level of expression is determined by the promoter, rather than by the stability of the mRNA, the level of the reporter mRNA can be measured directly, such as by Northern blot analysis.

[0245] Once activity is detected, mutational and/or deletional analyses may be employed to determine the minimal region and/or sequences required to initiate transcription. Thus, sequences can be deleted at the 5' end of the promoter region and/or at the 3' end of the promoter region, and nucleotide substitutions introduced. These constructs are then introduced to cells and their activity determined.

[0246] In one embodiment, the promoter may be a gamma zein promoter, an oleosin ole16 promoter, a globulins promoter, an actin I promoter, an actin cl promoter, a sucrose synthetase promoter, an INOPS promoter, an EXM5 promoter, a globulin2 promoter, a b-32, ADPG-pyrophosphorylase promoter, an Ltpl promoter, an Ltp2 promoter, an oleosin ole17 promoter, an oleosin ole18 promoter, an actin 2 promoter, a pollen-specific protein promoter, a pollen-specific pectate lyase promoter, an anther-specific protein promoter, an anther-specific gene RTS2 promoter, a pollen-specific gene promoter, a tapetum-specific gene promoter, tapetum-specific gene RAB24 promoter, an anthranilate synthase alpha subunit promoter, an alpha zein promoter, an anthranilate synthase beta subunit promoter, a dihydrodipicolinate synthase promoter, a Thil promoter, an alcohol dehydrogenase promoter, a cab binding protein promoter, an H3C4 promoter, a RUBISCO SS starch branching enzyme promoter, an ACCase promoter, an actin3 promoter, an actin7 promoter, a regulatory protein GF14-12 promoter, a ribosomal protein L9 promoter, a cellulose biosynthetic enzyme promoter, an S-adenosyl-L-homocysteine hydrolase promoter, a superoxide dismutase promoter, a C-kinase receptor promoter, a phosphoglycerate mutase promoter, a root-specific RCc3 mRNA promoter, a glucose-6 phosphate isomerase promoter, a pyrophosphate-fructose 6-phosphatophosphotransferase promoter, an ubiquitin promoter, a beta-ketoacyl-ACP synthase promoter, a 33 kDa photosystem 11 promoter, an oxygen evolving protein promoter, a 69 kDa vacuolar ATPase subunit promoter, a metallothionein-like protein promoter, a glyceraldehyde-3-phosphate dehydrogenase promoter, an ABA- and ripening-inducible-like protein promoter, a phenylalanine ammonia lyase promoter, an adenosine triphosphatase S-adenosyl-L-homocysteine hydrolase promoter, an a-tubulin promoter, a cab promoter,

a PEPCase promoter, an R gene promoter, a lectin promoter, a light harvesting complex promoter, a heat shock protein promoter, a chalcone synthase promoter, a zein promoter, a globulin-1 promoter, an ABA promoter, an auxin-binding protein promoter, a UDP glucose flavonoid glycosyl-transferase gene promoter, an NTI promoter, an actin promoter, an opaque 2 promoter, a b70 promoter, an oleosin promoter, a CaMV 35S promoter, a CaMV 34S promoter, a CaMV 19S promoter, a histone promoter, a turgor-inducible promoter, a pea small subunit RuBP carboxylase promoter, a Ti plasmid mannopine synthase promoter, Ti plasmid nopaline synthase promoter, a petunia chalcone isomerase promoter, a bean glycine rich protein I promoter, a CaMV 35S transcript promoter, a potato patatin promoter, or a S-E9 small subunit RuBP carboxylase promoter.

4. Transformed (Transgenic) Plants of the Invention and Methods of Preparation

[0247] Plant species may be transformed with the DNA construct of the present invention by the DNA-mediated transformation of plant cell protoplasts and subsequent regeneration of the plant from the transformed protoplasts in accordance with procedures well known in the art.

[0248] Any plant tissue capable of subsequent clonal propagation, whether by organogenesis or embryogenesis, may be transformed with a vector of the present invention. The term "organogenesis," as used herein, means a process by which shoots and roots are developed sequentially from meristematic centers; the term "embryogenesis," as used herein, means a process by which shoots and roots develop together in a concerted fashion (not sequentially), whether from somatic cells or gametes. The particular tissue chosen will vary depending on the clonal propagation systems available for, and best suited to, the particular species being transformed. Exemplary tissue targets include leaf disks, pollen, embryos, cotyledons, hypocotyls, megagametophytes, callus tissue, existing meristematic tissue (e.g., apical meristems, axillary buds, and root meristems), and induced meristem tissue (e.g., cotyledon meristem and utililane meristem).

[0249] Plants of the present invention may take a variety of forms. The plants may be chimeras of transformed cells and non-transformed cells; the plants may be clonal transformants (e.g., all cells transformed to contain the expression cassette); the plants may comprise grafts of transformed and untransformed tissues (e.g., a transformed root stock grafted to an untransformed scion in citrus species). The transformed plants may be propagated by a variety of means, such as by clonal propagation or classical breeding techniques. For example, first generation (or T1) transformed plants may be selfed to give homozygous second generation (or T2) transformed plants, and the T2 plants further propagated through classical breeding techniques. A dominant selectable marker (such as nptII) can be associated with the expression cassette to assist in breeding.

[0250] Thus, the present invention provides a transformed (transgenic) plant cell, *in planta* or *ex planta*, including a transformed plastid or other organelle, e.g., nucleus, mitochondria or chloroplast. The present invention may be used for transformation of any plant species, including, but not limited to, cells from the plant species specified above in the DEFINITION section. Preferably, transgenic plants of the present invention are crop plants and in particular cereals (for example, corn, alfalfa, sunflower, rice, Brassica, canola, soybean, barley, soybean, sugarbeet, cotton, safflower, peanut, sorghum, wheat, millet, tobacco, etc.), and even more preferably corn, rice and soybean. Other embodiments of the invention are related to cells, cell cultures, tissues, parts (such as plants organs, leaves, roots, etc.) and propagation material (such as seeds) of such plants.

[0251] The transgenic expression cassette of the invention may not only be comprised in plants or plant cells but may advantageously also be containing in other organisms such for example bacteria. Thus, another embodiment of the invention relates to transgenic cells or non-human, transgenic organisms comprising an expression cassette of the invention. Preferred are prokaryotic and eukaryotic organism. Both microorganism and higher organisms are comprised. Preferred microorganism are bacteria, yeast, algae, and fungi. Preferred bacteria are those of the genus *Escherichia*, *Erwinia*, *Agrobacterium*, *Flavobacterium*, *Alcaligenes*, *Pseudomonas*, *Bacillus* or *Cyanobacterim* such as - for example - *Synechocystis* and other bacteria described in Brock Biology of Microorganisms Eighth Edition (pages A-8, A-9, A10 and A11).

[0252] Especially preferred are microorganisms capable to infect plants and to transfer DNA into their genome, especially bacteria of the genus *Agrobacterium*, preferably *Agrobacterium tumefaciens* and *rhizogenes*. Preferred yeasts are *Candida*, *Saccharomyces*, *Hansenula* and *Pichia*. Preferred Fungi are *Aspergillus*, *Trichoderma*, *Ashbya*, *Neurospora*, *Fusarium*, and *Beauveria*. Most preferred are plant organisms as defined above.

[0253] Transformation of plants can be undertaken with a single DNA molecule or multiple DNA molecules (i.e., co-transformation), and both these techniques are suitable for use with the expression cassettes of the present invention. Numerous transformation vectors are available for plant transformation, and the expression cassettes of this invention can be used in conjunction with any such vectors. The selection of vector will depend upon the preferred transformation technique and the target species for transformation.

[0254] A variety of techniques are available and known to those skilled in the art for introduction of constructs into a plant cell host. These techniques generally include transformation with DNA employing *A. tumefaciens* or *A. rhizogenes* as the transforming agent, liposomes, PEG precipitation, electroporation, DNA injection, direct DNA uptake, micropro-

jectile bombardment, particle acceleration, and the like (See, for example, EP 295959 and EP 138341) (see below). However, cells other than plant cells may be transformed with the expression cassettes of the invention. The general descriptions of plant expression vectors and reporter genes, and *Agrobacterium* and *Agrobacterium*-mediated gene transfer, can be found in Gruber et al. (1993).

5 **[0255]** Expression vectors containing genomic or synthetic fragments can be introduced into protoplasts or into intact tissues or isolated cells. Preferably expression vectors are introduced into intact tissue. General methods of culturing plant tissues are provided for example by Maki et al., (1993); and by Phillips et al. (1988). Preferably, expression vectors are introduced into maize or other plant tissues using a direct gene transfer method such as microprojectile-mediated delivery, DNA injection, electroporation and the like. More preferably expression vectors are introduced into plant tissues using the microprojectile media delivery with the biolistic device. See, for example, Tomes et al. (1995). The vectors of the invention can not only be used for expression of structural genes but may also be used in exon-trap cloning, or promoter trap procedures to detect differential gene expression in varieties of tissues (Lindsey 1993; Auch & Reth 1990).

10 **[0256]** It is particularly preferred to use the binary type vectors of Ti and Ri plasmids of *Agrobacterium* spp. Ti-derived vectors transform a wide variety of higher plants, including monocotyledonous and dicotyledonous plants, such as soybean, cotton, rape, tobacco, and rice (Pacciotti 1985; Byrne 1987; Sukhapinda 1987; Lorz 1985; Potrykus, 1985; Park 1985; Hiei 1994). The use of T-DNA to transform plant cells has received extensive study and is amply described (EP 120516; Hoekema, 1985; Knauf, 1983; and An 1985). For introduction into plants, the chimeric genes of the invention can be inserted into binary vectors as described in the examples.

15 **[0257]** Other transformation methods are available to those skilled in the art, such as direct uptake of foreign DNA constructs (see EP 295959), techniques of electroporation (Fromm 1986) or high velocity ballistic bombardment with metal particles coated with the nucleic acid constructs (Kline 1987, and US 4,945,050). Once transformed, the cells can be regenerated by those skilled in the art. Of particular relevance are the recently described methods to transform foreign genes into commercially important crops, such as rapeseed (De Block 1989), sunflower (Everett 1987), soybean (McCabe 1988; Hinchee 1988; Chee 1989; Christou 1989; EP 301749), rice (Hiei 1994), and corn (Gordon-Kamm 1990; Fromm 1990).

20 **[0258]** Those skilled in the art will appreciate that the choice of method might depend on the type of plant, i.e., monocotyledonous or dicotyledonous, targeted for transformation. Suitable methods of transforming plant cells include, but are not limited to, microinjection (Crossway 1986), electroporation (Riggs 1986), *Agrobacterium*-mediated transformation (Hinchee 1988), direct gene transfer (Paszowski 1984), and ballistic particle acceleration using devices available from Agracetus, Inc., Madison, Wis. and BioRad, Hercules, Calif. (see, for example, US 4,945,050; and McCabe 1988). Also see, Weissinger 1988; Sanford 1987 (onion); Christou 1988 (soybean); McCabe 1988 (soybean); Datta 1990 (rice); Klein 1988 (maize); Klein 1988 (maize); Klein 1988 (maize); Fromm 1990 (maize); and Gordon-Kamm 1990 (maize); Svab 1990 (tobacco chloroplast); Koziel 1993 (maize); Shimamoto 1989 (rice); Christou 1991 (rice); European Patent Application EP 0 332 581 (orchardgrass and other Pooideae); Vasil 1993 (wheat); Weeks 1993 (wheat).

25 **[0259]** In another embodiment, a nucleotide sequence of the present invention is directly transformed into the plastid genome. Plastid transformation technology is extensively described in US 5,451,513, 5,545,817, and 5,545,818, in PCT application no. WO 95/16783, and in McBride et al., 1994. The basic technique for chloroplast transformation involves introducing regions of cloned plastid DNA flanking a selectable marker together with the gene of interest into a suitable target tissue, e.g., using biolistics or protoplast transformation (e.g., calcium chloride or PEG mediated transformation).

30 The 1 to 1.5 kb flanking regions, termed targeting sequences, facilitate orthologous recombination with the plastid genome and thus allow the replacement or modification of specific regions of the plastome. Initially, point mutations in the chloroplast 16S rRNA and rps12 genes conferring resistance to spectinomycin and/or streptomycin are utilized as selectable markers for transformation (Svab 1990; Staub 1992). This resulted in stable homoplasmic transformants at a frequency of approximately one per 100 bombardments of target leaves. The presence of cloning sites between these markers allowed creation of a plastid targeting vector for introduction of foreign genes (Staub 1993). Substantial increases in transformation frequency are obtained by replacement of the recessive rRNA or r-protein antibiotic resistance genes with a dominant selectable marker, the bacterial *aadA* gene encoding the spectinomycin-detoxifying enzyme aminoglycoside-3N-adenyltransferase (Svab 1993). Other selectable markers useful for plastid transformation are known in the art and encompassed within the scope of the invention. Typically, approximately 15-20 cell division cycles following transformation are required to reach a homoplasmic state. Plastid expression, in which genes are inserted by orthologous recombination into all of the several thousand copies of the circular plastid genome present in each plant cell, takes advantage of the enormous copy number advantage over nuclear-expressed genes to permit expression levels that can readily exceed 10% of the total soluble plant protein. In a preferred embodiment, a nucleotide sequence of the present invention is inserted into a plastid targeting vector and transformed into the plastid genome of a desired plant host. Plants homoplasmic for plastid genomes containing a nucleotide sequence of the present invention are obtained, and are preferentially capable of high expression of the nucleotide sequence.

35 **[0260]** *Agrobacterium tumefaciens* cells containing a vector comprising an expression cassette of the present invention, wherein the vector comprises a Ti plasmid, are useful in methods of making transformed plants. Plant cells are

infected with an *Agrobacterium tumefaciens* as described above to produce a transformed plant cell, and then a plant is regenerated from the transformed plant cell. Numerous *Agrobacterium* vector systems useful in carrying out the present invention are known.

[0261] Various *Agrobacterium* strains can be employed, preferably disarmed *Agrobacterium tumefaciens* or *rhizogenes* strains. In a preferred embodiment, *Agrobacterium* strains for use in the practice of the invention include octopine strains, e.g., LBA4404 or agropine strains, e.g., EHA101 or EHA105. Suitable strains of *A. tumefaciens* for DNA transfer are for example EHA101[pEHA101] (Hood 1986), EHA105[pEHA105] (Li 1992), LBA4404[pAL4404] (Hoekema 1983), C58C1[pMP90] (Koncz & Schell 1986), and C58C1[pGV2260] (Deblaere 1985). Other suitable strains are *Agrobacterium tumefaciens* C58, a nopaline strain. Other suitable strains are *A. tumefaciens* C58C1 (Van Larebeke 1974), A136 (Watson 1975) or LBA4011 (Klapwijk 1980). In another preferred embodiment the soil-borne bacterium is a disarmed variant of *Agrobacterium rhizogenes* strain K599 (NCPFB 2659). Preferably, these strains are comprising a disarmed plasmid variant of a Ti- or Ri-plasmid providing the functions required for T-DNA transfer into plant cells (e.g., the *vir* genes). In a preferred embodiment, the *Agrobacterium* strain used to transform the plant tissue pre-cultured with the plant phenolic compound contains a L,L-succinamopine type Ti-plasmid, preferably disarmed, such as pEHA101. In another preferred embodiment, the *Agrobacterium* strain used to transform the plant tissue pre-cultured with the plant phenolic compound contains an octopine-type Ti-plasmid, preferably disarmed, such as pAL4404. Generally, when using octopine-type Ti-plasmids or helper plasmids, it is preferred that the *virF* gene be deleted or inactivated (Jarschow 1991).

[0262] The method of the invention can also be used in combination with particular *Agrobacterium* strains, to further increase the transformation efficiency, such as *Agrobacterium* strains wherein the *vir* gene expression and/or induction thereof is altered due to the presence of mutant or chimeric *virA* or *virG* genes (e.g. Hansen 1994; Chen and Winans 1991; Scheeren-Groot, 1994). Preferred are further combinations of *Agrobacterium tumefaciens* strain LBA4404 (Hiei 1994) with super-virulent plasmids. These are preferably pTOK246-based vectors (Ishida 1996).

[0263] A binary vector or any other vector can be modified by common DNA recombination techniques, multiplied in *E. coli*, and introduced into *Agrobacterium* by e.g., electroporation or other transformation techniques (Mozo & Hooykaas 1991).

[0264] *Agrobacterium* is grown and used in a manner similar to that described in Ishida (1996). The vector comprising *Agrobacterium* strain may, for example, be grown for 3 days on YP medium (5 g/l yeast extract, 10 g/l peptone, 5 g/l NaCl, 15 g/l agar, pH 6.8) supplemented with the appropriate antibiotic (e.g., 50 mg/l spectinomycin). Bacteria are collected with a loop from the solid medium and resuspended. In a preferred embodiment of the invention, *Agrobacterium* cultures are started by use of aliquots frozen at - 80°C.

[0265] The transformation of the target tissue (e.g., an immature embryo) by the *Agrobacterium* may be carried out by merely contacting the target tissue with the *Agrobacterium*. The concentration of *Agrobacterium* used for infection and co-cultivation may need to be varied. For example, a cell suspension of the *Agrobacterium* having a population density of approximately from 10^5 - 10^{11} , preferably 10^6 to 10^{10} , more preferably about 10^8 cells or cfu / ml is prepared and the target tissue is immersed in this suspension for about 3 to 10 minutes. The resulting target tissue is then cultured on a solid medium for several days together with the *Agrobacterium*.

[0266] Preferably, the bacterium is employed in concentration of 10^6 to 10^{10} cfu/ml. In a preferred embodiment for the co-cultivation step about 1 to 10 μ l of a suspension of the soil-borne bacterium (e.g., *Agrobacteria*) in the co-cultivation medium are directly applied to each target tissue explant and air-dried. This is saving labor and time and is reducing unintended *Agrobacterium*-mediated damage by excess *Agrobacterium* usage.

[0267] For *Agrobacterium* treatment, the bacteria are resuspended in a plant compatible co-cultivation medium. Supplementation of the co-culture medium with antioxidants (e.g., silver nitrate), phenol-absorbing compounds (like polyvinylpyrrolidone, Perl 1996) or thiol compounds (e.g., dithiothreitol, L-cysteine, Olhoft 2001) which can decrease tissue necrosis due to plant defense responses (like phenolic oxidation) may further improve the efficiency of *Agrobacterium*-mediated transformation. In another preferred embodiment, the co-cultivation medium of comprises least one thiol compound, preferably selected from the group consisting of sodium thiosulfate, dithiothreitol (DTT) and cysteine. Preferably the concentration is between about 1 mM and 10mM of L-Cysteine, 0.1 mM to 5 mM DTT, and/or 0.1 mM to 5 mM sodium thiosulfate. Preferably, the medium employed during co-cultivation comprises from about 1 μ M to about 10 μ M of silver nitrate and from about 50 mg/L to about 1,000 mg/L of L-Cysteine. This results in a highly reduced vulnerability of the target tissue against *Agrobacterium*-mediated damage (such as induced necrosis) and highly improves overall transformation efficiency.

[0268] Various vector systems can be used in combination with *Agrobacteria*. Preferred are binary vector systems. Common binary vectors are based on "broad host range"-plasmids like pRK252 (Bevan 1984) or pTJS75 (Watson 1985) derived from the P-type plasmid RK2. Most of these vectors are derivatives of pBIN19 (Bevan 1984). Various binary vectors are known, some of which are commercially available such as, for example, pBI101.2 or pBIN19 (Clontech Laboratories, Inc. USA). Additional vectors were improved with regard to size and handling (e.g. pPZP; Hajdukiewicz 1994). Improved vector systems are described also in WO 02/00900.

[0269] Methods using either a form of direct gene transfer or *Agrobacterium*-mediated transfer usually, but not nec-

essarily, are undertaken with a selectable marker which may provide resistance to an antibiotic (e.g., kanamycin, hygromycin or methotrexate) or a herbicide (e.g., phosphinothricin). The choice of selectable marker for plant transformation is not, however, critical to the invention.

[0270] For certain plant species, different antibiotic or herbicide selection markers may be preferred. Selection markers used routinely in transformation include the nptII gene which confers resistance to kanamycin and related antibiotics (Messing & Vierra, 1982; Bevan 1983), the bar gene which confers resistance to the herbicide phosphinothricin (White 1990, Spencer 1990), the hph gene which confers resistance to the antibiotic hygromycin (Blochlinger & Diggelmann), and the dhfr gene, which confers resistance to methotrexate (Bourouis 1983).

5. Production and Characterization of Stably Transformed Plants

[0271] Transgenic plant cells are then placed in an appropriate selective medium for selection of transgenic cells which are then grown to callus. Shoots are grown from callus and plantlets generated from the shoot by growing in rooting medium. The various constructs normally will be joined to a marker for selection in plant cells. Conveniently, the marker may be resistance to a biocide (particularly an antibiotic, such as kanamycin, G418, bleomycin, hygromycin, chloramphenicol, herbicide, or the like). The particular marker used will allow for selection of transformed cells as compared to cells lacking the DNA which has been introduced. Components of DNA constructs including transcription cassettes of this invention may be prepared from sequences which are native (endogenous) or foreign (exogenous) to the host. By "foreign" it is meant that the sequence is not found in the wild-type host into which the construct is introduced. Heterologous constructs will contain at least one region which is not native to the gene from which the transcription-initiation-region is derived.

[0272] To confirm the presence of the transgenes in transgenic cells and plants, a variety of assays may be performed. Such assays include, for example, "molecular biological" assays well known to those of skill in the art, such as Southern and Northern blotting, in situ hybridization and nucleic acid-based amplification methods such as PCR or RT-PCR; "biochemical" assays, such as detecting the presence of a protein product, e.g., by immunological means (ELISAs and Western blots) or by enzymatic function; plant part assays, such as seed assays; and also, by analyzing the phenotype of the whole regenerated plant, e.g., for disease or pest resistance.

[0273] DNA may be isolated from cell lines or any plant parts to determine the presence of the preselected nucleic acid segment through the use of techniques well known to those skilled in the art. Note that intact sequences will not always be present, presumably due to rearrangement or deletion of sequences in the cell.

[0274] The presence of nucleic acid elements introduced through the methods of this invention may be determined by polymerase chain reaction (PCR). Using this technique discrete fragments of nucleic acid are amplified and detected by gel electrophoresis. This type of analysis permits one to determine whether a preselected nucleic acid segment is present in a stable transformant, but does not prove integration of the introduced preselected nucleic acid segment into the host cell genome. In addition, it is not possible using PCR techniques to determine whether transformants have exogenous genes introduced into different sites in the genome, i.e., whether transformants are of independent origin. It is contemplated that using PCR techniques it would be possible to clone fragments of the host genomic DNA adjacent to an introduced preselected DNA segment.

[0275] Positive proof of DNA integration into the host genome and the independent identities of transformants may be determined using the technique of Southern hybridization. Using this technique specific DNA sequences that were introduced into the host genome and flanking host DNA sequences can be identified. Hence the Southern hybridization pattern of a given transformant serves as an identifying characteristic of that transformant. In addition it is possible through Southern hybridization to demonstrate the presence of introduced preselected DNA segments in high molecular weight DNA, i.e., confirm that the introduced preselected, DNA segment has been integrated into the host cell genome. The technique of Southern hybridization provides information that is obtained using PCR, e.g., the presence of a preselected DNA segment, but also demonstrates integration into the genome and characterizes each individual transformant.

[0276] It is contemplated that using the techniques of dot or slot blot hybridization which are modifications of Southern hybridization techniques one could obtain the same information that is derived from PCR, e.g., the presence of a preselected DNA segment.

[0277] Both PCR and Southern hybridization techniques can be used to demonstrate transmission of a preselected DNA segment to progeny. In most instances the characteristic Southern hybridization pattern for a given transformant will segregate in progeny as one or more Mendelian genes (Spencer 1992); Laursen 1994) indicating stable inheritance of the gene. The non-chimeric nature of the callus and the parental transformants (R_0) was suggested by germline transmission and the identical Southern blot hybridization patterns and intensities of the transforming DNA in callus, R_0 plants and R_1 progeny that segregated for the transformed gene.

[0278] Whereas DNA analysis techniques may be conducted using DNA isolated from any part of a plant, RNA may only be expressed in particular cells or tissue types and hence it will be necessary to prepare RNA for analysis from these tissues. PCR techniques may also be used for detection and quantitation of RNA produced from introduced

preselected DNA segments. In this application of PCR it is first necessary to reverse transcribe RNA into DNA, using enzymes such as reverse transcriptase, and then through the use of conventional PCR techniques amplify the DNA. In most instances PCR techniques, while useful, will not demonstrate integrity of the RNA product. Further information about the nature of the RNA product may be obtained by Northern blotting. This technique will demonstrate the presence of an RNA species and give information about the integrity of that RNA. The presence or absence of an RNA species can also be determined using dot or slot blot Northern hybridizations. These techniques are modifications of Northern blotting and will only demonstrate the presence or absence of an RNA species.

[0279] While Southern blotting and PCR may be used to detect the preselected DNA segment in question, they do not provide information as to whether the preselected DNA segment is being expressed. Expression may be evaluated by specifically identifying the protein products of the introduced preselected DNA segments or evaluating the phenotypic changes brought about by their expression.

[0280] Assays for the production and identification of specific proteins may make use of physical-chemical, structural, functional, or other properties of the proteins. Unique physical-chemical or structural properties allow the proteins to be separated and identified by electrophoretic procedures, such as native or denaturing gel electrophoresis or isoelectric focusing, or by chromatographic techniques such as ion exchange or gel exclusion chromatography. The unique structures of individual proteins offer opportunities for use of specific antibodies to detect their presence in formats such as an ELISA assay. Combinations of approaches may be employed with even greater specificity such as Western blotting in which antibodies are used to locate individual gene products that have been separated by electrophoretic techniques. Additional techniques may be employed to absolutely confirm the identity of the product of interest such as evaluation by amino acid sequencing following purification. Although these are among the most commonly employed, other procedures may be additionally used.

[0281] Assay procedures may also be used to identify the expression of proteins by their functionality, especially the ability of enzymes to catalyze specific chemical reactions involving specific substrates and products. These reactions may be followed by providing and quantifying the loss of substrates or the generation of products of the reactions by physical or chemical procedures. Examples are as varied as the enzyme to be analyzed.

[0282] Very frequently the expression of a gene product is determined by evaluating the phenotypic results of its expression. These assays also may take many forms including but not limited to analyzing changes in the chemical composition, morphology, or physiological properties of the plant. Morphological changes may include greater stature or thicker stalks. Most often changes in response of plants or plant parts to imposed treatments are evaluated under carefully controlled conditions termed bioassays.

6. Uses of Transgenic Plants

[0283] Once an expression cassette of the invention has been transformed into a particular plant species, it may be propagated in that species or moved into other varieties of the same species, particularly including commercial varieties, using traditional breeding techniques. Particularly preferred plants of the invention include the agronomically important crops listed above. The genetic properties engineered into the transgenic seeds and plants described above are passed on by sexual reproduction and can thus be maintained and propagated in progeny plants. The present invention also relates to a transgenic plant cell, tissue, organ, seed or plant part obtained from the transgenic plant. Also included within the invention are transgenic descendants of the plant as well as transgenic plant cells, tissues, organs, seeds and plant parts obtained from the descendants.

[0284] Preferably, the expression cassette in the transgenic plant is sexually transmitted. In one preferred embodiment, the coding sequence is sexually transmitted through a complete normal sexual cycle of the R0 plant to the R1 generation. Additionally preferred, the expression cassette is expressed in the cells, tissues, seeds or plant of a transgenic plant in an amount that is different than the amount in the cells, tissues, seeds or plant of a plant which only differs in that the expression cassette is absent.

[0285] The transgenic plants produced herein are thus expected to be useful for a variety of commercial and research purposes. Transgenic plants can be created for use in traditional agriculture to possess traits beneficial to the grower (e.g., agronomic traits such as resistance to water deficit, pest resistance, herbicide resistance or increased yield), beneficial to the consumer of the grain harvested from the plant (e.g., improved nutritive content in human food or animal feed; increased vitamin, amino acid, and antioxidant content; the production of antibodies (passive immunization) and nutraceuticals), or beneficial to the food processor (e.g., improved processing traits). In such uses, the plants are generally grown for the use of their grain in human or animal foods. Additionally, the use of root-specific promoters in transgenic plants can provide beneficial traits that are localized in the consumable (by animals and humans) roots of plants such as carrots, parsnips, and beets. However, other parts of the plants, including stalks, husks, vegetative parts, and the like, may also have utility, including use as part of animal silage or for ornamental purposes. Often, chemical constituents (e.g., oils or starches) of maize and other crops are extracted for foods or industrial use and transgenic plants may be created which have enhanced or modified levels of such components.

[0286] Transgenic plants may also find use in the commercial manufacture of proteins or other molecules, where the molecule of interest is extracted or purified from plant parts, seeds, and the like. Cells or tissue from the plants may also be cultured, grown in vitro, or fermented to manufacture such molecules. The transgenic plants may also be used in commercial breeding programs, or may be crossed or bred to plants of related crop species. Improvements encoded

by the expression cassette may be transferred, e.g., from maize cells to cells of other species, e.g., by protoplast fusion. [0287] The transgenic plants may have many uses in research or breeding, including creation of new mutant plants through insertional mutagenesis, in order to identify beneficial mutants that might later be created by traditional mutation and selection. An example would be the introduction of a recombinant DNA sequence encoding a transposable element that may be used for generating genetic variation. The methods of the invention may also be used to create plants having

unique "signature sequences" or other marker sequences which can be used to identify proprietary lines or varieties. [0288] Thus, the transgenic plants and seeds according to the invention can be used in plant breeding which aims at the development of plants with improved properties conferred by the expression cassette, such as tolerance of drought, disease, or other stresses. The various breeding steps are characterized by well-defined human intervention such as selecting the lines to be crossed, directing pollination of the parental lines, or selecting appropriate descendant plants.

Depending on the desired properties different breeding measures are taken. The relevant techniques are well known in the art and include but are not limited to hybridization, inbreeding, backcross breeding, multilane breeding, variety blend, interspecific hybridization, aneuploid techniques, etc. Hybridization techniques also include the sterilization of plants to yield male or female sterile plants by mechanical, chemical or biochemical means. Cross pollination of a male sterile plant with pollen of a different line assures that the genome of the male sterile but female fertile plant will uniformly obtain properties of both parental lines. Thus, the transgenic seeds and plants according to the invention can be used for the breeding of improved plant lines which for example increase the effectiveness of conventional methods such as herbicide or pesticide treatment or allow to dispense with said methods due to their modified genetic properties. Alternatively new crops with improved stress tolerance can be obtained which, due to their optimized genetic "equipment", yield harvested product of better quality than products which were not able to tolerate comparable adverse developmental conditions.

EXAMPLES

Materials and General Methods

[0289] Unless indicated otherwise, chemicals and reagents in the Examples were obtained from Sigma Chemical Company (St. Louis, MO), restriction endonucleases were from New England Biolabs (Beverly, MA) or Roche (Indianapolis, IN), oligonucleotides were synthesized by MWG Biotech Inc. (High Point, NC), and other modifying enzymes or kits regarding biochemicals and molecular biological assays were from Clontech (Palo Alto, CA), Pharmacia Biotech (Piscataway, NJ), Promega Corporation (Madison, WI), or Stratagene (La Jolla, CA). Materials for cell culture media were obtained from Gibco/BRL (Gaithersburg, MD) or DIFCO (Detroit, MI). The cloning steps carried out for the purposes of the present invention, such as, for example, restriction cleavages, agarose gel electrophoresis, purification of DNA fragments, transfer of nucleic acids to nitrocellulose and nylon membranes, linking DNA fragments, transformation of *E. coli* cells, growing bacteria, multiplying phages and sequence analysis of recombinant DNA, are carried out as described by Sambrook (1989). The sequencing of recombinant DNA molecules is carried out using ABI laser fluorescence DNA sequencer following the method of Sanger (Sanger 1977).

[0290] For generating transgenic *Arabidopsis* plants *Agrobacterium tumefaciens* (strain C58C1[pMP90]) is transformed with the various promoter::GUS vector constructs (see below). Resulting *Agrobacterium* strains are subsequently employed to obtain transgenic plants. For this purpose a isolated transformed *Agrobacterium* colony is incubated in 4 ml culture (Medium: YEB medium with 50 µg/ml Kanamycin and 25 µg/ml Rifampicin) over night at 28°C. With this culture a 400 ml culture of the same medium is inoculated and incubated over night (28 °C, 220 rpm). The bacteria a precipitated by centrifugation (GSA-Rotor, 8.000 U/min, 20 min) and the pellet is resuspended in infiltration medium (1/2 MS-Medium; 0,5 g/l MES, pH 5,8; 50 g/l sucrose). The suspension is placed in a plant box (Duchefa) and 100 ml SILVET L-77 (Osi Special-ties Inc., Cat. P030196) are added to a final concentration of 0.02%. The plant box with 8 to 12 Plants is placed into an exsiccator for 10 to 15 min. under vacuum with subsequent, spontaneous ventilation (expansion). This process is repeated 2-3 times. Thereafter all plants are transferred into pods with wet-soil and grown under long daytime conditions (16 h light; day temperature 22-24°C, night temperature 19°C; 65% rel. humidity). Seeds are harvested after 6 weeks.

EXAMPLE 1: Growth conditions for plants for tissue-specific expression analysis

[0291] To obtain 4 and 7 days old seedlings, about 400 seeds (*Arabidopsis thaliana* ecotype Columbia) are sterilized with a 80% (v/v) ethanol:water solution for 2 minutes, treated with a sodium hypochlorite solution (0.5% v/v) for 5 minutes, washed three times with distilled water and incubated at 4°C for 4 days to ensure a standardized germination. Sub-

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sequently, seeds are incubated on Petri dishes with MS medium (Sigma M5519) supplemented with 1% sucrose, 0.5 g/l MES (Sigma M8652), 0.8% Difco-BactoAgar (Difco 0140-01), adjusted to pH 5.7. The seedlings are grown under 16 h light / 8 h dark regime (Philips 58W/33 white light) at 22°C and harvested after 4 or 7 days, respectively.

[0292] To obtain root tissue, 100 seeds are sterilized as described above, incubated at 4°C for 4 days, and transferred into 250ml flasks with MS medium (Sigma M5519) supplemented with additional 3% sucrose and 0.5 g/l MES (Sigma M8652), adjusted to pH 5.7 for further growing. The seedlings are grown at a 16 h light / 8 h dark cycle (Philips 58W/33 white light) at 22°C and 120 rpm and harvested after 3 weeks. For all other plant organs employed, seeds are sown on standard soil (Type VM, Manna-Italia, Via S. Giacomo 42, 39050 San Giacomo/ Laives, Bolzano, Italy), incubated for 4 days at 4°C to ensure uniform germination, and subsequently grown under a 16 h light / 8 h darkness regime (OSRAM Lumi-lux Daylight 36W/12) at 22°C. Young rosette leaves are harvested at the 8-leaf stage (after about 3 weeks), mature rosette leaves are harvested after 8 weeks briefly before stem formation. Apices of out-shooting stems are harvested briefly after out-shooting. Stem, stem leaves, and flower buds are harvested in development stage 12 (Bowmann J (ed.), *Arabidopsis*, Atlas of Morphology, Springer New York, 1995) prior to stamen development. Open flowers are harvested in development stage 14 immediately after stamen development. Wilting flowers are harvested in stage 15 to 16. Green and yellow shoots used for the analysis have a length of 10 to 13 mm.

EXAMPLE 2: Demonstration of expression profile

[0293] To demonstrate and analyze the transcription regulating properties of a promoter of the useful to operably link the promoter or its fragments to a reporter gene, which can be employed to monitor its expression both qualitatively and quantitatively. Preferably bacterial β -glucuronidase is used (Jefferson 1987). β -glucuronidase activity can be monitored *in planta* with chromogenic substrates such as 5-bromo-4-Chloro-3-indolyl- β -D-glucuronic acid during corresponding activity assays (Jefferson 1987). For determination of promoter activity and tissue specificity plant tissue is dissected, embedded, stained and analyzed as described (e.g., Bäumlein 1991).

[0294] For quantitative β -glucuronidase activity analysis MUG (methylumbelliferyl glucuronide) is used as a substrate, which is converted into MU (methylumbelliferone) and glucuronic acid. Under alkaline conditions this conversion can be quantitatively monitored fluorometrically (excitation at 365 nm, measurement at 455 nm; SpectroFluorimeter Thermo Life Sciences Fluoroscan) as described (Bustos 1989).

EXAMPLE 3: Cloning of the promoter fragments

[0295] To isolate the promoter fragments described by SEQ ID NO: 1, 2, 3, 4, 5, 6, 7, 8, 9, 12, 13, 14, and 15, genomic DNA is isolated from *Arabidopsis thaliana* (ecotype Columbia) as described (Galbiati 2000). The isolated genomic DNA is employed as template DNA for a polymerase chain reaction (PCR) mediated amplification using the oligonucleotide primers and protocols indicated below (Table 3).

Table 3: PCR conditions and oligonucleotide primers for amplification of the various transcription regulating nucleotide sequences

SEQ ID	Promoter	Forward Primer	Reverse Primer	Restriction enzymes
SEQ ID NO: 1	pSUH305			EcoRI/EcoRI
SEQ ID NO: 2	pSUH305S	SUH305Sfor SEQ ID NO: 16	SUH305Srev SEQ ID NO: 17	EcoRI/NcoI
SEQ ID NO: 3	pSUH305GB	SUH305Sfor SEQ ID NO: 16	SUH305Srev SEQ ID NO: 17	EcoRI/NcoI
SEQ ID NO: 4	pSUK132	SUK132for SEQ ID NO: 18	SUK132rev SEQ ID NO: 19	BamHI/NcoI
SEQ ID NO: 5	pSUK132GB	SUK132for SEQ ID NO: 18	SUK132rev SEQ ID NO: 19	BamHI/NcoI
SEQ ID NO: 6	pSUK134	SUK134for SEQ ID NO: 20	SUK134rev SEQ ID NO: 21	BamHI/NcoI
SEQ ID NO: 7	pSUK134GB	SUK134for SEQ ID NO: 20	SUK134rev SEQ ID NO: 21	BamHI/NcoI

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(continued)

SEQ ID	Promoter	Forward Primer	Reverse Primer	Restriction enzymes
SEQ ID NO: 8	pSUK136	SUK136for SEQ ID NO: 22	SUK136rev SEQ ID NO: 23	BamHI/NcoI
SEQ ID NO: 9	pSUK136GB	SUK136for SEQ ID NO: 22	SUK136rev SEQ ID NO: 23	BamHI/NcoI
SEQ ID NO: 12	pSUK342	SUK342for SEQ ID NO: 24	SUK342rev SEQ ID NO: 25	BamHI/NcoI
SEQ ID NO: 13	pSUK342GB	SUK342for SEQ ID NO: 24	SUK342rev SEQ ID NO: 25	BamHI/NcoI
SEQ ID NO: 14	pSUK344	SUK344for SEQ ID NO: 26	SUK344rev SEQ ID NO: 27	BamHI/NcoI
SEQ ID NO: 15	pSUK344GB	SUK344for SEQ ID NO: 26	SUK344rev SEQ ID NO: 27	BamHI/NcoI

[0296] Amplification is carried out as follows:

100 ng genomic DNA
 1X PCR buffer
 2,5 mM MgCl₂,
 200 μM each of dATP, dCTP, dGTP und dTTP
 10 pmol of each oligonucleotide primers
 2,5 Units Pfu DNA Polymerase (Stratagene)
 in a final volume of 50 μl

[0297] The following temperature program is employed for the various amplifications (BIORAD Thermocycler).

1. 95°C for 5 min
2. 54°C for 1 min, followed by 72°C for 5 min and 95°C for 30 sec. Repeated 25 times
3. 54°C for 1 min, followed by 72°C for 10min.
4. Storage at 4°C

[0298] The transcription regulating nucleotide sequence as described by SEQ ID NO: 1 (designated pSUH305) is a chimeric sequence comprising a transcription regulating nucleotide sequence from *Arabidopsis thaliana* (as described by SEQ ID NO: 2) and a short stretch of sequence derived from the 3' terminus of the transposon Ds. Base pair 1 to 931 of pSUH305 are representing genomic DNA of *Arabidopsis thaliana*, base pair 932 to 1001 are representing the Ds transposon element/primer binding site as part of a transposon-based promoter trap system (based on *Ac/Ds*-elements (*Activator/Dissociation*); Martienssen, 1998).

[0299] The chimeric sequence was constructed by ligating the PCR amplified transcription regulating nucleotide sequence described by SEQ ID NO: 2 to the stretch of Ds transposon as described above using standard cloning techniques.

[0300] The resulting PCR-products are digested with the restriction endonucleases specified in the table above (Table 3) and cloned into the vector pSUN0301 (SEQ ID NO: 28) (pre-digested with the same enzymes) upstream and in operable linkage to the glucuronidase (GUS) gene. Following stable transformation of each of these constructs into *Arabidopsis thaliana* tissue specificity and expression profile was analyzed by a histochemical and quantitative GUS-assay, respectively.

EXAMPLE 4: Expression profile of the various promoter::GUS constructs in stably transformed *A. thaliana* plants

4.1 pSUH305, pSUH305S, pSUH305GB

[0301] These promoters are active in and highly specific for guard cells of all organs analyzed in seedlings as well as in adult plants.

4.2 pSUK132, pSUK132GB, pSUK134, pSUK134GB, pSUK136, pSUK136GB

[0302] These promoter fragments drive guard cell-preferential expression in all organs analyzed. Side activity of promoter::reporter gene constructs was observed in mesophyll cells, vascular tissue of leaves and stalks as well as in roots (especially in root tips).

4.3 pSUK342, pSUK342GB, pSUK344, pSUK344GB

[0303] The promoter is active in guard cells of leaves and stalks. Side activity was observed in mesophyll tissue and in roots of seedlings.

EXAMPLE 5 Vector Construction for Overexpression and Gene "Knockout" Experiments

5.1 Overexpression

[0304] Vectors used for expression of full-length "candidate genes" of interest in plants (overexpression) are designed to overexpress the protein of interest and are of two general types, biolistic and binary, depending on the plant transformation method to be used.

[0305] For biolistic transformation (biolistic vectors), the requirements are as follows:

1. a backbone with a bacterial selectable marker (typically, an antibiotic resistance gene) and origin of replication functional in *Escherichia coli* (*E. coli*; e.g., ColE1), and
2. a plant-specific portion consisting of:
 - a. a gene expression cassette consisting of a promoter (e.g. ZmUBlint MOD), the gene of interest (typically, a full-length cDNA) and a transcriptional terminator (e.g., *Agrobacterium tumefaciens* nos terminator);
 - b. a plant selectable marker cassette, consisting of a suitable promoter, selectable marker gene (e.g., D-amino acid oxidase; *dao1*) and transcriptional terminator (e.g. nos terminator).

[0306] Vectors designed for transformation by *Agrobacterium tumefaciens* (*A. tumefaciens*; binary vectors) consist of:

1. a backbone with a bacterial selectable marker functional in both *E. coli* and *A. tumefaciens* (e.g., spectinomycin resistance mediated by the *aadA* gene) and two origins of replication, functional in each of aforementioned bacterial hosts, plus the *A. tumefaciens* *virG* gene;
2. a plant-specific portion as described for biolistic vectors above, except in this instance this portion is flanked by *A. tumefaciens* right and left border sequences which mediate transfer of the DNA flanked by these two sequences to the plant.

5.2 Gene Silencing Vectors

[0307] Vectors designed for reducing or abolishing expression of a single gene or of a family or related genes (gene silencing vectors) are also of two general types corresponding to the methodology used to downregulate gene expression: antisense or double-stranded RNA interference (dsRNAi).

(a) Anti-sense

[0308] For antisense vectors, a full-length or partial gene fragment (typically, a portion of the cDNA) can be used in the same vectors described for full-length expression, as part of the gene expression cassette. For antisense-mediated down-regulation of gene expression, the coding region of the gene or gene fragment will be in the opposite orientation relative to the promoter; thus, mRNA will be made from the non-coding (antisense) strand in planta.

(b) dsRNAi

[0309] For dsRNAi vectors, a partial gene fragment (typically, 300 to 500 base pairs long) is used in the gene expression cassette, and is expressed in both the sense and antisense orientations, separated by a spacer region (typically, a plant intron, eg the *OsSH1* intron 1, or a selectable marker, e.g. conferring kanamycin resistance). Vectors of this type are

designed to form a double-stranded mRNA stem, resulting from the base pairing of the two complementary gene fragments *in planta*.

[0310] Biolistic or binary vectors designed for overexpression or knockout can vary in a number of different ways, including eg. the selectable markers used in plant and bacteria, the transcriptional terminators used in the gene expression and plant selectable marker cassettes, and the methodologies used for cloning in gene or gene fragments of interest (typically, conventional restriction enzyme-mediated or Gateway™ recombinase-based cloning).

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[0312] All publications, patents and patent applications are incorporated herein by reference. While in the foregoing
 55 specification this invention has been described in relation to certain preferred embodiments thereof, and many details
 have been set forth for purposes of illustration, it will be apparent to those skilled in the art that the invention is susceptible
 to additional embodiments and that certain of the details described herein may be varied considerably without departing
 from the basic principles of the invention.

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<220>
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 <222> (1)..(933)
 <223> transcription regulating sequence

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 <213> Arabidopsis thaliana

<220>
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 <223> transcription regulating sequence of gene At5g58580

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<210> 5
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 <213> Arabidopsis thaliana

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<210> 6
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<213> Arabidopsis thaliana

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<222> (1)..(1009)
<223> transcription regulating sequence of gene At5g58580

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catttcatca atattccaaa caatgattat gcatgtaatc catatatata attttttttt 720
attcataagg aattatacat tggtcctcaa tattattttc aggttcaaga atgagtttag 780
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gtaattttga ttatccctat actatacact tttactctag acacatgtat atagttggcc 960
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<210> 7
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<220>
<221> promoter

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<222> (1)..(1023)
 <223> transcription regulating sequence of gene At5g58580

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<220>
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 30 <223> transcription regulating sequence of gene At5g58580

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<223> transcription regulating sequence of gene At5g58580

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 <213> Arabidopsis thaliana

25 <220>
 <221> CDS
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 <223> encoding zinc finger (C3HC4-type RING finger) family protein

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	Ser Phe Leu Lys Ile Leu Ser Ser Tyr Asn Ser Asn Val Leu Leu Ala	
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35	gct cta gtc ttc ctt ctc tta gtc gtc ctc ttc gtc ttg ctt ctc cat	144
	Ala Leu Val Phe Leu Leu Leu Val Val Leu Phe Val Leu Leu Leu His	
	35 40 45	
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	Phe Tyr Ala Arg Phe Phe Trp Ser Pro Ser His Gln Asp Phe Ser Ala	
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	Ala Ala Arg His Arg Arg Arg Arg Arg Arg Asn Arg Arg Arg Thr Val	
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	Thr Thr Thr Arg Ile Ile Pro Ser Leu Pro Leu Gly Gly Phe Asp Asp	
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	Gly Val Ser Ser Pro Ala Ala Thr Ala Thr Arg Asp Asp Lys Gly Leu	
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	Asp Ser Ser Val Ile Ser Ser Ile Pro Leu Phe Val Tyr Glu Glu Asn	
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	Glu Glu Glu Glu Asp Glu Glu Glu Glu Cys Val Ile Cys Leu Gly Leu	
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 Phe His Val Glu Cys Ile Asp Met Trp Leu Ser Ser His Ser Thr Cys
 165 170 175
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 180 185 190
 10 ctt aag ctg gcc gtt aac gcc gtc gaa gaa gaa gca gag gtt aga tta 624
 Leu Lys Leu Ala Val Asn Ala Val Glu Glu Glu Ala Glu Val Arg Leu
 195 200 205
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 Gln Met Ser Pro Ala Gly Glu Asn Glu Ser Asn Val Ser Gly Asp Arg
 210 215 220
 15 aga gtt tca ctg tct ctt tct gta atg gaa gat gat ctc aaa aca ggg 720
 Arg Val Ser Leu Ser Leu Ser Val Met Glu Asp Asp Leu Lys Thr Gly
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 Glu Glu Ile Asn Asp Gly Gly Thr Arg Ser Asp Arg Arg Arg Ser Met
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 35 40 45
 45 Phe Tyr Ala Arg Phe Phe Trp Ser Pro Ser His Gln Asp Phe Ser Ala
 50 55 60
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 65 70 75 80
 50 Thr Thr Thr Arg Ile Ile Pro Ser Leu Pro Leu Gly Gly Phe Asp Asp
 85 90 95
 Gly Val Ser Ser Pro Ala Ala Thr Ala Thr Arg Asp Asp Lys Gly Leu
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 55

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Asp Ser Ser Val Ile Ser Ser Ile Pro Leu Phe Val Tyr Glu Glu Asn
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 Glu Glu Glu Glu Asp Glu Glu Glu Glu Cys Val Ile Cys Leu Gly Leu
 130 135 140
 Trp Glu Ala Gly Asp Phe Gly Arg Lys Leu Arg Asn Cys Gly His Gly
 145 150 155 160
 10
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Claims

- 15 **1.** An expression cassette for regulating guard cell-preferential or guard cell-specific expression in plants comprising
- a) at least one transcription regulating nucleotide sequence selected from the group consisting of
- i) the sequences described by SEQ ID NOs: 1, 2 and 3
- 20 ii) a fragment of at least 50 consecutive bases of a sequence under i) which has substantially the same promoter activity as the corresponding transcription regulating nucleotide sequence described by SEQ ID NO: 1, 2 or 3,;
- iii) a nucleotide sequence having substantial similarity with a sequence identity of at least 40% to a transcription regulating nucleotide sequence described by SEQ ID NO: 1, 2 or 3;
- 25 iv) a nucleotide sequence capable of hybridizing under conditions equivalent to hybridization in 7% sodium dodecyl sulfate (SDS), 0.5 M NaPO₄, 1 mM EDTA at 50°C with washing in 2 X SSC, 0.1% SDS at 50°C to a transcription regulating nucleotide sequence described by SEQ ID NO: 1, 2 or 3 or the complement thereof;
- 30 v) a nucleotide sequence capable of hybridizing under conditions equivalent to hybridization in 7% sodium dodecyl sulfate (SDS), 0.5 M NaPO₄, 1 mM EDTA at 50°C with washing in 2 X SSC, 0.1% SDS at 50°C to a nucleic acid comprising 50 to 200 or more consecutive nucleotides of a transcription regulating nucleotide sequence described by SEQ ID NO: 1, 2 or 3 or the complement thereof;
- vi) a nucleotide sequence which is the complement or reverse complement of any of the previously mentioned nucleotide sequences under i) to v).
- 35 and functionally linked thereto
- b) at least one nucleic acid sequence which is heterologous in relation to said transcription regulating nucleotide sequence.
- 40 **2.** The expression cassette of Claim 1, wherein expression of the nucleic acid sequence results in expression of a protein, or expression of an antisense RNA, sense or double-stranded RNA.
- 3.** The expression cassette of any of Claim 1 to 2, wherein expression of the nucleic acid sequence confers to the plant an agronomically valuable trait.
- 45 **4.** A vector comprising an expression cassette of any of Claim 1 to 3.
- 5.** A transgenic host cell or non-human organism comprising an expression cassette of any of Claim 1 to 3, or a vector of Claim 4.
- 50 **6.** A transgenic plant comprising the expression cassette of any of Claim 1 to 3, a vector of Claim 4, or a cell of claim 5.

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EUROPEAN SEARCH REPORT

Application Number
EP 09 17 5928

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (IPC)
A	DE 199 04 754 A1 (MAX PLANCK GESELLSCHAFT [DE]) 10 August 2000 (2000-08-10) * abstract; col. 1-7 and 19-24; Figures 1 and 4 *	1-6	INV. C12N15/82 C12N5/10 C12N15/29 C07K14/415 C12Q1/68 A01H5/00
A	----- PLESCH GUNNAR ET AL: "Involvement of TAAAG elements suggests a role for Dof transcription factors in guard cell-specific gene expression" PLANT JOURNAL, vol. 28, no. 4, November 2001 (2001-11), pages 455-464, XP002367787 ISSN: 0960-7412 * abstract; Figures 3 and 5; pages 456-460 *	1-6	
A	----- DATABASE EMBL [Online] 27 August 1999 (1999-08-27), "Arabidopsis thaliana chromosome I BAC F22D16 genomic sequence, complete sequence." XP002564607 retrieved from EBI accession no. EMBL:AC009525 Database accession no. AC009525 * Nucleotides 69209-70141compound *	1-6	TECHNICAL FIELDS SEARCHED (IPC) C12N C12Q C07K
A	----- PLESCH<1> G ET AL: "Cloning of regulatory sequences mediating guard-cell-specific gene expression" GENE, ELSEVIER, AMSTERDAM, NL, vol. 249, no. 1-2, 1 May 2000 (2000-05-01) , pages 83-89, XP004199729 ISSN: 0378-1119 * abstract; pages 87 and 88; Table 1 *	1-6	
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The present search report has been drawn up for all claims			
Place of search Munich		Date of completion of the search 21 January 2010	Examiner Kurz, Birgit
CATEGORY OF CITED DOCUMENTS X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document		T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons ----- & : member of the same patent family, corresponding document	

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EUROPEAN SEARCH REPORT

Application Number
EP 09 17 5928

DOCUMENTS CONSIDERED TO BE RELEVANT			
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A	<p>WU K ET AL: "Enhancers and core promoter elements are essential for the activity of a cryptic gene activation sequence from tobacco, tCUP" MGG MOLECULAR GENETICS AND GENOMICS, vol. 265, no. 5, 31 March 2001 (2001-03-31), pages 763-770, XP002564608 ISSN: 1617-4615 * abstract; pages 763-764, 767-769; Figure 1A *</p> <p style="text-align: center;">-----</p>	1-6	
			TECHNICAL FIELDS SEARCHED (IPC)
The present search report has been drawn up for all claims			
1	Place of search Munich	Date of completion of the search 21 January 2010	Examiner Kurz, Birgit
<p>CATEGORY OF CITED DOCUMENTS</p> <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p>		<p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document</p>	

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**ANNEX TO THE EUROPEAN SEARCH REPORT
ON EUROPEAN PATENT APPLICATION NO.**

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This annex lists the patent family members relating to the patent documents cited in the above-mentioned European search report. The members are as contained in the European Patent Office EDP file on
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21-01-2010

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For more details about this annex : see Official Journal of the European Patent Office, No. 12/82

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